American *Type Culture* Collection

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Fusarium equiseti **(continued)**

†44413 L Hornok 22-107. Alfalfa root, Hungary. Taxonomy (Trans. Br. Mycol. Soc. *74:* 73-78, 1980). (Medium 338 24C) **Shipped:** Test tube.

†46469 G. Morgan-Jones. *Heterodera glycines* cysts, soybean field, Missouri. Isolation (Nematropica *11:* 155-163, 1981). (Medium 338 24C) **Shipped:** Freeze-dried.

†56090 A. Bottalico ITM-192. Durum wheat stem, Italy. (Medium 338 25C under light 12 hrs on/12 hrs off) **Shipped:** Freeze-dried.

†60276 M. Baxter MU 1006 — H.M. Hussein. Maize husk, New Zealand. (Medium 338 26C under black light) **Shipped:** Test tube.

†60316 G.A. Neish 80.023 — G. Platford. [DAOM 180360] Strawberry field soil, Canada. (Medium 338 26C under black light) **Shipped:** Test tube.

†60383 NRRL 5909. (Medium 338 24C) **Shipped:** Freeze-dried.

†62721 R.G. Roberts 84-41. Sunflower seed, North Carolina. Isolation (Can. J. Bot. *64:* 1964-1971, 1986). (Medium 336 24C) **Shipped:** Freeze-dried.

Fusarium flocciferum Corda

24371 CMI 13515 — W. Gams. Wheat field soil, Germany. (Medium 338 24C) **Shipped:** Freeze-dried.

Fusarium fusarioides (Fragoso *et* Ciferri) Booth: See also *Dactylium fusarioides,* teleomorph

24372 CMI 128101 — S.I. Ahmend. [CBS 124.73] Soil, Pakistan. (Medium 338 24C) **Shipped:** Freeze-dried.

62725 R.G. Roberts 84-154. Sunflower seed, Georgia. Isolation (Can. J. Bot. *64:* 1964-1971, 1986). (Medium 336 24C) **Shipped:** Freeze-dried.

Fusarium graminearum Schwabe: See also *Gibberella zeae,* teleomorph

†15624 W.L. Gordon 2611-SA 1153. Corn stalk, Canada. (Medium 336 26C) **Shipped:** Freeze-dried.

†20329 RHM Res. Ltd. I 7/3 — G. Scammell. United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654 and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 336 30C) **Shipped:** Test tube.

†20330 RHM Res. Ltd. I 8/3 — G. Scammell. Derived from parent strain IO after 1100 hrs culture, United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654 and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 338 30C) **Shipped:** Test tube.

†20331 RHM Res. Ltd. I 9/2 — G. Scammell. Derived from parent strain IO after 1100 hrs culture, United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654 and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 338 30C) **Shipped:** Test tube.

†20332 RHM Res. Ltd. I 15/2 — G. Scammell. Derived from parent strain IO after 1100 hrs culture, United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654, and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 323 30C) **Shipped:** Test tube.

†20333 RHM Res. Ltd. I 16/2 — G. Scammell. Derived from parent strain IO after 1100 hrs culture, United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654 and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 323 30C) **Shipped:** Test tube.

†20334 RHM Res. Ltd. I 0/5 — G. Scammell. [CMI 145425] Soil, United Kingdom. Production of edible protein substances (U.S. Pats. 3,937,654 and 3,937,693). **Note:** This material is cited in a U.S. and/or other Patent and may not be used to infringe the patent claims. (Medium 323 30C) **Shipped:** Freeze-dried.

†24373 CMI 160243 — P. Martin. [CBS 316.73] *Zea mays,* South Africa. (Medium 338 24C) **Shipped:** Freeze-dried.

†26557 CMI 155426 — C.J. Mirocha. *Zea mays* moldy grain, USA. Produces zearalenone (F-2 toxin) (J. Stored Prod. Res. *8:* 71-75, 1972). (Medium 336 24C) **Shipped:** Test tube.

†34909 M. Palyusik F 59. [NRRL 6392] Millet, Hungary. Produces mycotoxins (Appl. Environ. Microbiol. *32:* 579-584, 1976). (Medium 338 24C) **Shipped:** Freeze-dried.

†34912 M. Palyusik F 184a. [NRRL 6394] Millet, Hungary. Produces mycotoxins (Appl. Environ. Microbiol. *32:* 579-584, 1976). (Medium 338 24C) **Shipped:** Freeze-dried.

†36882 E.L. Hintikka 72192 — H. Heiskanen. Barley, Finland. (Medium 335 26C) **Shipped:** Test tube.

†36884 E.L. Hintikka 72299 — H. Heiskanen. Barley, Finland. (Medium 335 26C) **Shipped:** Freeze-dried.

†36885 E.L. Hintikka 72322 — H. Heiskanen. Wheat, Finland. (Medium 335 26C) **Shipped:** Freeze-dried.

†44418 L. Hornok 22-39. Winter wheat seed, Hungary. Taxonomy (Trans. Br. Mycol. Soc. *74:* 73-78, 1980). (Medium 338 24C) **Shipped:** Test tube.

†46779 NRRL 5883. Corn. Produces vomitoxin (Appl. Environ. Microbiol. *43:* 967-970, 1982). (Medium 336 24C) **Shipped:** Freeze-dried.

†56091 A. Bottalico ITM-124. Rice stem, Italy. (Medium 338 25C under light 12 hrs on/12 hrs off) **Shipped:** Freeze-dried.

†56092 A. Bottalico ITM-126. Soft wheat stem, Italy. (Medium 338 25C under light 12 hrs on/12 hrs off) **Shipped:** Freeze-dried.

†56093 A. Bottalico ITM-127. Maize ear, Italy. (Medium 338 25C) **Shipped:** Freeze-dried.

†56748 NRRL 5864. Produces zearalenone. (Medium 336 24C) **Shipped:** Freeze-dried.

†56749 NRRL 6012. Produces moniliformin. (Medium 336 24C) **Shipped:** Freeze-dried.

†58667 K. Kallela 7137-18 — H. Heiskanan. Fodder, Finland. Produces zearalenone (Nord. Veterinaermed. *33:* 454-460, 1981). (Medium 307 26C under light 12 hrs on/12 hrs off) **Shipped:** Test tube.

†60289 M. Baxter MU 1011 — H.M. Hussein. Maize grain, New Zealand. (Medium 338 26C under black light) **Shipped:** Test tube.

†60309 G.A. Neish GAN 905-7-A. [DAOM 177410] White winter wheat, Canada. Produces vomitoxin (Can. J. Plant Sci. *61:* 811-815, 1981) and zearalenone *(ibid.;* Can. J. Plant Pathol. *4:* 191-194, 1982). (Medium 338 26C under black light) **Shipped:** Freeze-dried.

†60643 J. Chelkowski KF 208. (Medium 336 26C) **Shipped:** Test tube.

†60880 M. Baxter MU 1027 — H. Hussein. Maize grain, New Zealand. (Medium 338 24C under black light) **Shipped:** Test tube.

†60881 M. Baxter MU 1028 — H. Hussein. Maize grain, New Zealand. (Medium 338 24C under black light) **Shipped:** Test tube.

Fusarium heterosporum Nees

†15625 W.L. Gordon 3396. Raspberry, Scotland. Heterothallic. Mating type A. (Medium 336 26C) **Shipped:** Test tube.

†15626 W.L. Gordon 2883-SA 37. Heterothallic. Mating type a. (Medium 336 26C) **Shipped:** Test tube.

†15627 W.L. Gordon 2883 — P. Salisbury. Canker on white pine, Canada. Heterothallic. Mating type a. (Medium 336 26C) **Shipped:** Freeze-dried.

†15628 W.L. Gordon 2857-SA 89. Heterothallic. Mating type A. (Medium 336 26C) **Shipped:** Freeze-dried.

†15629 W.L. Gordon 2798 — J. Sibalis. Cankered rose twig, Canada. Heterothallic. Mating type A. (Medium 336 26C) **Shipped:** Test tube.

†32872 B.M. Cunfer F73-1. Ergot honeydew, Georgia. Sphacelial stage of *Claviceps purpurea* and *C. paspali.* Possible biological control, capable of preventing sclerotial formation (Phytopathology *65:* 1372-1374, 1975; *ibid., 66:* 449-452, 1976). (Medium 336 15-25C) **Shipped:** Freeze-dried.

†44419 L. Hornok 22-115. Winter wheat leaf, Hungary. Taxonomy (Trans. Br. Mycol. Soc. *74:* 73-78, 1980). (Medium 338 24C) **Shipped:** Test tube.

†48715 A.S. Foudin. *Festuca arundinacea,* Montana. Head-scab (Plant Dis. *66:* 866, 1982). (Medium 338 28C under light) **Shipped:** Freeze-dried.

†52540 W.F.O. Marasas MRC 1816. [Gerlach 62229] (Medium 338 24C) **Shipped:** Freeze-dried.

†USDA

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Article No. FG971027

Species-Specific Primers Resolve Members of *Fusarium* Section *Fusarium*

Taxonomic Status of the Edible "Quorn" Fungus Reevaluated

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Yoder, W. T., and Christianson, L. M. 1998. Species-Specific Primers Resolve Members of *Fusarium* Section *Fusarium*. Taxonomic Status of the Edible "Quorn" Fungus Reevaluated. *Fungal Genetics and Biology.* 23, 68–80. Sixty-seven authentic isolates, representing six species from *Fusarium* section *Fusarium* (= section Discolor) were subjected to random amplified polymorphic DNA (RAPD) analysis and polymerase chain reaction using species-specific primers. Remarkably uniform RAPD banding patterns were obtained intraspecifically, irrespective of the geographical origin of the isolates or the host/substratum from which they were isolated. Isolates were also assessed for colony characteristics when grown on a defined minimal medium. The Quorn strain (ATCC 20334; previously considered to be *F. graminearum*) matched the *F. venenatum* strains exclusively in RAPD profile. In addition, equivalently sized DNA fragments amplified from ATCC 20334 and two authentic *F. venenatum* strains were identical with respect to DNA sequence. Our molecular and morphological data support the identification of the Quorn strain as *F. venenatum* Nirenberg (= *F. sambucinum* Fuckel *sensu lato*). © 1998 Academic Press

Index Descriptors: RAPDs; *Fusarium*; *Fusarium venenatum*; species-specific primers; fungal taxonomy; Quorn; identification.

Fusarium species, which are mitosporic, have a cosmopolitan distribution and can infect a wide variety of plant species. Despite the fact that members of this genus represent economically important plant pathogens, identification to species level is not always straightforward. This is because the cultural and morphological appearance of *Fusarium* strains can be highly variable depending on the culture conditions employed and also because the different taxonomic systems published for the genus (all of which are based on cultural and morphological characteristics) describe different numbers of sections, species, and varieties. For example, Nelson *et al.* (1983) recognize 30 species and varieties, Booth (1971) 51, and Gerlach and Nirenberg (1982) 101.

The *Fusarium* strain ATCC 20334 has been used for more than 10 years to produce Quorn mycoprotein (marketed by Marlow Foods in England). This product is sold for human consumption in the United Kingdom as a high-fiber, low-fat food with a protein content similar to that of whole eggs and a texture resembling that of lean meat (Trinci, 1992). The *Fusarium* strain ATCC 20334 was originally isolated from soil in the United Kingdom and was deposited as *F. graminearum* in a number of international culture depositaries [e.g., American Type Culture Collection, MD (Accession No. ATCC 20334), the Imperial Mycological Institute, Surrey, UK (Accession No. 145425), the Agriculture Canada and Agri-Food Canada Culture Collection (Accession No. DAOM 193459), and the Northern Regional Research Laboratories, Peoria, IL (Accession No. NRRL 25417)]. In addition the strain was cited as *F. graminearum* in both UK (e.g., 1346061 and 1346062) and U.S. patents (e.g., 3937653 and 3937694).

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Since *Fusarium* strain ATCC 20334 has also been shown to be useful as a host for heterologous protein expression (Royer *et al.*, 1995), verification of its taxonomic status was desirable. The Quorn strain, ATCC 20334, was therefore submitted for identification to several independent taxonomic authorities. The strain was subsequently identified (on the basis of morphology and cultural characteristics) as *F. sulphureum* Schlechtendahl (= *F. sambucinum* Fuckel), *F. crookwellense* Burgess *et al.*, and *F. venenatum* Nirenberg. These species are all considered to belong to the section *Fusarium* [which is equivalent to the section Discolor (Wollenweber and Reinking, 1935; Nelson *et al.*, 1983)], in which 21 species are recognized by Gerlach and Nirenberg (1982) and 6 by Nelson *et al.* (1983).

One important member of the section *Fusarium* is *F. sambucinum* (s.l.), which occurs in soil and on many plants in temperate and subtropoical zones. In particular *F. sambucinum* (s.l.) strains can cause a dry rot of potato tubers in the field and in storage. Current taxonomic views on this species differ widely (as discussed by Nirenberg, 1995) and in order to reduce these different views, collaborators of the European *F. sambucinum* project initiated morphological, molecular, secondary metabolite, and mating behavior analyses. The results of these investigations (Nirenberg, 1995; Hering and Nirenberg, 1995; Logrieco *et al.*, 1995; Thrane and Hansen, 1995; Desjardins and Nelson, 1995) provided strong support for the differentiation of *F. sambucinum* (s.l.) into three species—*F. sambucinum* Fuckel (s.s.), *F. torulosum* (Berk. & Curt.) Nirenberg, and *F. venenatum*.

Given the diversity of opinion we encountered among the taxonomic experts, morphology as the sole criterion was not considered adequate to allow accurate identification of ATCC 20334, which is a deteriorated, pionnotal mutant. We therefore employed RAPDs[2] (and species-specific primers) in addition to cultural morphology on a defined minimal medium (MM) to compare ATCC 20334 with multiple authentic isolates of six species belonging to the section *Fusarium* (obtained from the *Fusarium* Research Center, Pennsylvania State University, University Park, and from Biologische Bundesanstalt fur Land- und Fortswirtschaft, Institute of Microbiology, Berlin). RAPDs were chosen because they have been developed and applied successfully as species level indicators in the section *Fusarium*, i.e., to identify *F. graminearum* and *F. culmorum* strains (Schilling, 1996) and to distinguish *F. sambucinum* (s.s.), *F. torulosum* (Berk. & M.A. Curt.) Nirenberg, and *F. venenatum* strains (Hering and Nirenberg, 1995). Apart from the fact that gene cloning and sequencing are obviated, the use of RAPDs as species indicators allows accurate analysis of deteriorated, nonsporulating, and pionnotal mutants since the method is independent of gene expression. Cultural morphology and linear extension rates of all isolates on a defined MM were also assessed, since the major *Fusarium* taxonomic systems currently in use are based on morphological and cultural criteria (e.g., Nelson *et al.*, 1983; Booth 1971, 1977; Gerlach and Nirenberg, 1982; Wollenweber and Reinking, 1935). The use of a defined minimal medium for morphological comparisons was considered pertinent, since growth under stressful conditions may be expected to exacerbate differences between individuals to a greater extent than growth on rich medial. Our hypothesis was that species within the section *Fusarium* should be consistently resolvable on the basis of molecular comparisons of whole genomes and that the data generated using such an approach should correlate with cultural characteristics.

A preliminary report of these data was presented at the 19th Fungal Genetics Conference held at Asilomar, California, March 18–23, 1997.

[2] Abbreviations used: RAPD, random amplified polymorphic DNA; MM, minimal medium; LER, linear extension rate.

MATERIALS AND METHODS

Media

Minimal medium. Per liter—glucose 25 g, 10 mM urea, Noble agar 25 g, MM salts 20 ml, pH 6. MM salts: Per liter—KCl 26 g, $MgSO_4 \cdot 7H_2O$ 26 g, KH_2PO_4 76 g, trace metals 50 ml. MM trace metals: Per liter—$Na_2B_4O_7 \cdot 10H_2O$ 40 mg, $CuSO_4 \cdot 5H_2O$ 400 mg, $FeSO_4 \cdot 7H_2O$ 1.2 g, $MnSO_4 \cdot H_2O$ 700 mg, $Na_2MoO_2 \cdot 2H_2O$ 800 mg, $ZnSO_4 \cdot 7H_2O$ 10 g.

YPG. Per liter—yeast extract 10 g, peptone 20 g, and glucose 25 g.

Sporulation medium. Per liter—50× sporulation salts 20 ml, sodium nitrate 12.1 g, succinic acid, disodium salt 50 g, glucose 1 g. 50× sporulation salts: Per liter—Na_3 citrate $\cdot 2H_2O$ 125 g, KH_2PO_4 250 g, $MgSO_4 \cdot 7H_2O$ 10 g, $CaCl_2 \cdot 2H_2O$ 5 g (predissolved in 20 ml water), trace elements solution 5 ml, biotin solution 2.5 ml. Trace elements: Per 100 ml—citric acid $\cdot H_2O$ 5 g, $ZnSO_4 \cdot 7H_2O$ 5 g, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.25 g, $MnSO_4H_2O$ 0.05 g, H_3BO_3 0.05 g, $Na_2MoO_4 \cdot 2H_2O$ 0.05 g. Biotin: 5 mg in 100 ml 50% (v/v) ethanol.

TABLE 1
Fusarium Strains Used for RAPD Analysis

No.	Species	Host/substrate	Source	Original ID no.
23	*Fusarium* species	Soil, UK	ATCC	20334
1	*F. graminearum*	Maize, New Zealand	ATCC	60881
2	*F. graminearum*	Maize, Italy	ATCC	56093
46	*F. graminearum*	Corn, AZ	FRC	R-6629
49	*F. graminearum*	Barley, PA	FRC	R-6857
50	*F. graminearum*	Soil, MI	FRC	R-4728
52	*F. graminearum*	Barley, Canada	FRC	R-8426
53	*F. graminearum*	Fern, Netherlands	FRC	R-6238
54	*F. graminearum*	*Triticale*, Poland	FRC	R-7768
55	*F. graminearum*	Soil debris, S. Africa	FRC	R-8650
48	*F. graminearum?*	Wheat, WY	FRC	R-6009
51	*F. graminearum?*	Soil, NSW, Australia	FRC	R-6221
78	*Gibberella zeae*	Unknown	ATCC	10910
7	*F. crookwellense*	Potato tuber, Poland	ATCC	66007
8	*F. crookwellense*	Wheat, S. Africa	ATCC	64237
25	*F. crookwellense*	Turf, PA	FRC	R-1247
30	*F. crookwellense*	Turf, NY	FRC	R-4006
31	*F. crookwellense*	Alfalfa, PA	FRC	R-4151
32	*F. crookwellense*	Soil, MI	FRC	R-4746
33	*F. crookwellense*	Soil, MI	FRC	R-4758
47	*F. crookwellense*[a]	Turf, NY	FRC	R-4023
56	*F. crookwellense*	Soil, Australia	FRC	R-6683
57	*F. crookwellense*	Wheat, Canada	FRC	R-7162
58	*F. crookwellense*	Soil, China	FRC	R-7376
59	*F. crookwellense*	Cereal, Poland	FRC	R-8277
60	*F. crookwellense*	Soil debris, S. Africa	FRC	R-7224
62	*F. crookwellense*[b]	Corn, Canada	FRC	R-6354
41	*F. culmorum*	Potato tuber, ID	FRC	R-2635
43	*F. culmorum*	Red clover, WI	FRC	R-5183
44	*F. culmorum*	Oat stem base, CA	FRC	R-5209
66	*F. culmorum*	Pasture soil, Australia	FRC	R-5383
67	*F. culmorum*	Barley, Canada	FRC	R-7043
68	*F. culmorum*	Barley, Denmark	FRC	R-8504
69	*F. culmorum*	Wheat, Poland	FRC	R-7771
42	*F. culmorum?*[c]	Wheat, MN	FRC	R-5183
70	*F. culmorum?*	Soil, South Africa	FRC	R-8195
76	*Gibberella pulicaris*[d]	Potato, Germany	FRC	R-6380
77	*Gibberella pulicaris*[d]	Cabbage, Netherlands	BBA	64995
13	*F. sambucinum* (s.s.)[e]	Potato, ID	ATCC	44653
14	*F. sambucinum* (s.s.)[e]	*Solanum nigrum*, South Africa	ATCC	52543
35	*F. sambucinum* (s.s.)[e]	Potato, ID	FRC	R-2633
36	*F. sambucinum* (s.s.)[e]	Corn, MN	FRC	R-5455
39	*F. sambucinum* (s.s.)[e]	Potato, PA	FRC	R-9322
63	*F. sambucinum* (s.s.)[e]	Knotweed, England	FRC	R-583
65	*F. sambucinum* (s.s.)[e]	Carnation, Chile	FRC	R-7843
81	*F. sambucinum* (s.s.)	Potato, Germany	BBA	62397
82	*F. sambucinum* (s.s.)	Potato, UK	BBA	64226
83	*F. sambucinum* (s.s.)	Potato, Germany	BBA	64737
84	*F. sambucinum* (s.s.)	*Pterocarya*, Iran	BBA	62719
61	*F. torulosum*[f]	*Pinus radiata*, Australia	FRC	R-5920
85	*F. torulosum*[g]	*Beta vulgaris*, Germany	BBA	62396
86	*F. torulosum*	*Pinus*, Australia	BBA	65417
87	*F. torulosum*	*Hortensia*, Germany	BBA	65962
88	*F. torulosum*	Soil, Poland	BBA	66833
89	*F. torulosum*	*Triticum*, Australia	BBA	63933
90	*F. torulosum*	*Betula*, Germany	BBA	62398
10	*F. venenatum*[h]	Maize, New Zealand	ATCC	60879
64	*F. venenatum*[i]	Soil, South Africa	FRC	R-8135
92	*F. venenatum*	Potato, Finland	BBA	64478
93	*F. venenatum*	*Triticum aestivum*, Austria	BBA	64537
94	*F. venenatum*	*Humulus lupulus*, Germany	BBA	64757
95	*F. venenatum*	Potato, Poland	BBA	64935
96	*F. venenatum*	*Zea mays*, Germany	BBA	65030
98	*F. venenatum*	Soil, Germany	BBA	67619
99	*F. venenatum*	Cereal, France	BBA	67657
100	*F. venenatum*	Cucumber, Austria	BBA	68425
91	*F. venenatum?*	*Cucurbita maxima*, New Zealand	BBA	64376
97	*F. venenatum?*	Spinach, Sweden	BBA	65893

Note. ATCC, American Type Culture Collection, MD; FRC, *Fusarium* Research Center, Pennsylvania State University, University Park; BBA, Biologische Bundesanstalt fur Land- und Fortswirtschaft, Berlin, Germany.

[a] Initially received as *F. graminearum*, but RAPDs and morphology on MM and the conclusion of Dr. H. Nirenberg suggest identity with *F. crookwellense*.

[b] Received as *F. sambucinum* (s.l.) but RAPDs and morphology on MM suggest identity with *F. crookwellense*.

[c] RAPDs suggest that this is not a typical *F. culmorum* isolate. Dr. H. Nirenberg has identified it as *F. sporotrichioides* Sherb. (pers. comm.).

[d] Kindly provided by Anne Desjardins, USDA, Peoria, IL.

[e] Originally received as *F. sambucinum* (s.l.)—RAPDs and comparisons with authentic *F. sambucinum* (s.s.) strains suggest these isolates are all *F. sambucinum* (s.s.).

[f] Initially received as *F. sambucinum* (s.l.), but RAPDs and independent identification (H. Nirenberg, pers. comm.) suggest identity with *F. torulosum* (see also Fig. 4).

[g] Received as *F. sambucinum (s.str.)*, but our RAPD and morphological data suggest identity with *F. torulosum* strains.

[h] Initially received as *F. crookwellense* (ATCC) and confirmed as such by FRC, but identified as *F. venenatum* (H. Nirenberg, pers. comm.). RAPDs and morphology on MM support the *F. venenatum* designation.

[i] Initially received as *F. sambucinum* (s.l.), but RAPDs and independent morphological identification (H. Nirenberg, pers. comm.) suggest identity with *F. venenatum* (see also Fig. 4).

Fungal Strains

The strains used in this study are listed in Table 1. Importation of all strains was covered under USDA PPQ 526/599 permits. On receipt, all strains were revived on MM and incubated at 28°C. They were stored as plugs (cut from agar plates) in 10% glycerol (v/v) at −140°C.

DNA Extraction

Glass baffled shake flasks (125 ml) containing 25 ml YPG were inoculated in duplicate with four agar plugs (4 × 4 mm) of each strain and incubated for 72 h at 28°C and 150 rpm. After incubation, mycelia were harvested through sterile Miracloth (Calbiochem, San Diego, CA) and rinsed three times with 25 ml sterile distilled water. Mycelia were squeezed dry using a sterile glove and stored in sterile 1.7-ml Eppendorf tubes at −80°C prior to processing.

Mycelia were ground in liquid nitrogen using a pestle and mortar, and a small amount of the finely ground powder was transferred to a sterile 0.65-ml Eppendorf tube (to the 0.2-ml line marker on the side of the tube). Five hundred microliters of lysis buffer (100 mM EDTA, 10 mM Tris (pH 8), 1% Triton X-100, 500 mM guanidine HCl, and 200 mM NaCl) were added to the tube and mixed before RNase was added to a final concentration of 20 μg/ml. The samples were incubated in a waterbath at 37°C for 30 min. Proteinase K was added to a final concentration of 0.1 mg/ml and the samples were incubated in a waterbath at 50°C for 2 h. Samples were centrifuged at full speed for 15 min and the supernatants applied to Qiaprep Spin columns (Qiagen Kit 27104). DNA was washed and eluted according to the manufacturer's instructions.

DNA concentration was estimated by running 5 μl of each prep on a 1% agarose gel run in TAE buffer and comparing band intensities with different dilutions of λ *Hin*dIII-cut DNA.

RAPD PCR Amplifications

Oligonucleotide primers from Operon Technology Kit OPW selected for use in the current study were OPW-01, CTCAGTGTCC; OPW-02, ACCCCGCCAA; OPW-03, GTCCGGAGTG; OPW-04, CAGAAGCGGA; OPW-05, GGCGGATAAG; OPW-06, AGGCCCGATG; OPW-07, CTGGACGTCA; OPW-15, ACACCGGAAC; OPW-17, GTCCTGGGTT; and OPW-19, CAAAGCGCTC.

All polymerase chain reactions contained 50 mM KCl; 10 mM Tris–HCl, pH 8; 1.5 mM $MgCl_2$; 100 mM each dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech); 25 pmol primer (Operon Technology); 25 ng genomic DNA; 2.5 U *Taq* DNA polymerase (Boehringer Mannheim); and sterile distilled water to a total volume of 50 μl. Two drops of mineral oil were added to overlay the mixture in each tube.

All reactions were performed using a Stratagene Robocycler 40. The program was 1 cycle of 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C.

Gel Electrophoresis

Ten microliters of each DNA sample was loaded with 2 μl 6× running buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% (w/v) sucrose in H_2O) on a 1.8% agarose gel (1× TBE containing 0.5 μg/ml ethidium bromide) with 2 μl Boehringer Mannheim Molecular Weight Marker X. Electrophoresis was performed for 2 h at 100 V. After the gels were destained in water for 45 min, DNA was visualized using an Ultralum UV transilluminator.

Cloning of the VEN-A, VEN-B, and VEN-C Bands from *F. venenatum*

RAPD reactions using primer OPW-05 with DNA from the authentic *F. venenatum* strains and ATCC 20334 all generated a strong band of approximately 276 bp in length. This band, designated VEN-B, was cloned and sequenced from two separate *F. venenatum* strains (Nos. 92 and 93) and from ATCC 20334 as described below.

After excision of the 276-bp bands from 1% agarose gels, the DNA was purified and reamplified by using 1 μl of purified fragment as template under the same conditions as the original RAPD amplification. Each reamplified PCR product was ligated into pCR2.1 from the TA Cloning Kit (Invitrogen, San Diego, CA) following the manufacturer's instructions. Competent *Escherichia coli* "One Shot" cells (Invitrogen) were transformed with 2 μl of each ligation reaction and plated onto 2XYT plates with 100 μg/ml ampicillin. DNA from transformants was prepared using QIAprep8 (Qiagen, Inc.) and digested with *Eco*RI to identify clones that contained the VEN-B insert.

Two amplified fragments from each strain (six total) were sequenced using the M13 −20 forward and −48 reverse primers on an Applied Biosystems Model 373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). All templates were sequenced twice with the indicated primers. Sequences were analyzed using Autoassembler (Applied Biosystems, Inc.) and Lasergene (DNASTAR, Inc., Madison, WI).

Two other bands, VEN-A and VEN-C, were also isolated by PCR using primers OPW-03 (850 bp) and OPW-15 (2000 bp), respectively, and their DNA sequences were compared.

Design of Species-Specific Primers

Unique, species-specific RAPD bands were identified from two different isolates of each of the six species. Each

band was purified (Qiaquick Gel Extraction Kit; Qiagen) and reamplified using the original RAPD primer to confirm that the target fragment had been isolated. After ligation into pCR2.1 (TA Cloning Kit; Invitrogen) and transformation into *E. coli* DH5α (Gibco BRL, Fredrick, MD), plasmid DNA from the resulting transformants was analyzed by restriction digestion to identify those containing appropriately sized bands. For each band, sequences from at least two isolates were obtained. Primers were designed based on the 3′ and 5′ ends of the sequenced fragments and were either inclusive or exclusive primers. Inclusive primers included the original RAPD 10-mer primer, and exclusive primers began just 3′ of the RAPD primer sequence at each end of the fragment (see Table 2). Each primer pair was tested with DNA from at least four different isolates of each species using the PCR amplification conditions described above, except that the annealing temperature was 60°C instead of 35°C. Of 19 primer pairs tested, 6 were ultimately chosen for use. The sequences of these 6 primer pairs were as follows: CRO-A, CTCAGTGTCCACCGCGTTGCGTAG and CTCAGTGTCCCAATCAAATAGTCC; CRO-C, TATTGGGATCTATCCAAGTCTTGT and AAGCAGGAAACAGAAACCCTTTCC; CUL-A, TTTCAGCGGGCAACTTTGGGTAGA and AAGCTGAAATACGCGGTTGATAGG; SAM-E, CAGAAGCGGAGCAAGTTCACAATC and CAGAAGCGGATGGAGATGTAAAGT; TOR-B, CAAAGCGCTCCCTCAATCTCGTAC and CAAAGCGCTCATCAACTCCATATA; and VEN-B, GGCGGATAAGGATAGTGGTAGAAG and GGCGGATAAGCAAATAAGATGCTT.

Morphological Characteristics and Linear Extension Rates (LERs) on Minimal Medium

All isolates were inoculated by cutting 4 × 4 mm plugs submarginally from 7-day-old colonies on MM plates and placing these onto the centers of fresh MM plates. These were incubated in Chex-All bags (Propper, NY; Cat. No. 024012) at 28°C (12 h light/12 h dark) for up to 10 days. During this time the positions of colony margins were marked on the undersides of the petri dishes on four radii, at the same time every day. Linear extension rates (cm/day) were calculated from these measurements for all isolates.

Other morphological characters scored at the conclusion of the incubation period included the pigmentation of the upper and lower surfaces of the colonies, a description of the type (e.g., farinaceous versus velvety), the amount and distribution of aerial mycelia, and an assessment of the degree of sectoring evident (if any).

TABLE 2
Details of Species-Specific Primers

Primer pair	OPW primer	Amplified band size (bp)	OPW sequences included?
CRO-A	01	842	Yes
CRO-C	06	897	No
CUL-A	01	380	No
SAM-E	04	312	Yes
TOR-B	19	664	Yes
VEN-B	05	276	Yes

RESULTS

RAPD Analyses

The primers selected for use in the present study generated remarkably consistent species-specific banding patterns, despite the fact that the experimental strains had been deliberately selected from diverse geographical locations and a wide range of different hosts and substrata (Fig. 1). This observation was crucial to our objective of identifying strains of known affinity which generated RAPD banding patterns similar to the Quorn strain, ATCC 20334. However, 6 of the 67 strains included in this study clearly bore a much higher degree of similarity to other species on the basis of their RAPD banding patterns, PCR results with species-specific primers, and morphological characteristics (Table 1 and Fig. 1). Thus *F. graminearum* 47 was more similar to *F. crookwellense*, *F. crookwellense* 10 was more similar to *F. venenatum*, *F. sambucinum* (s.str.) 85 was more similar to *F. torulosum*, *F. sambucinum* (s.l.) 62 was more similar to *F. crookwellense*, *F. sambucinum* (s.l.) 61 was more similar to *F. torulosum* (Fig. 4), and *F. sambucinum* (s.l.) 64 was more similar to *F. venenatum*.

An additional six strains did not generate the RAPD banding patterns expected on the basis of those generated by all other members of the same species. *F. culmorum* strains 42 and 70, *F. graminearum* strains 48 and 51, and *F. venenatum* strains 91 and 97 were consistently different from all other members of their respective species with respect to RAPD banding patterns and morphology on MM. These strains could not be assigned to any of the other species included in this study. Strain 42 was subsequently identified as *F. sporotrichiodes* Sherb. on the basis

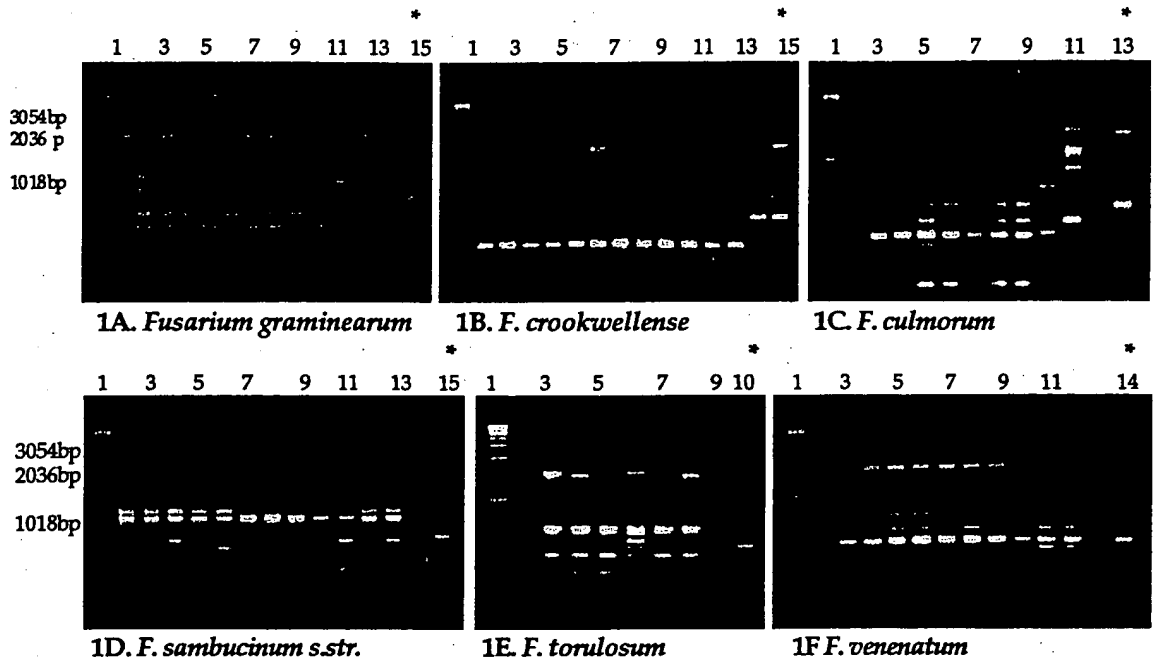


FIG. 1. RAPDs generated using primer OPW-03 and DNA from ATCC 20334 (*) and isolates of *F. graminearum* (A), *F. crookwellense* (B), *F. culmorum* (C), *F. sambucinum* (s.str.) (D), *F. torulosum* (E), and *F. venenatum* (F). (A) *F. graminearum* isolates. Lane 1, Boehringer Mannheim DNA marker X; lane 2, *Gibberella zeae* 78; lane 3, *F. graminearum* 46; lane 4, 49; lane 5, 50; lane 6, 52; lane 7, 53; lane 8, 54; lane 9, 55; lane 10, 1; lane 11, 2; lane 12, 48; lane 13, 51; lane 14, empty; lane 15, ATCC 20334 (*). (B) *F. crookwellense* isolates. Lane 1, Boehringer Mannheim DNA marker X; lane 2, 25; lane 3, 30; lane 4, 31; lane 5, 32; lane 6, 33; lane 7, 56; lane 8, 57; lane 9, 58; lane 10, 59; lane 11, 60; lane 12, 7; lane 13, No. 38; lane 14, 10; lane 15, ATCC 20334 (*). (C) *F. culmorum*. Lane 1, Boehringer Mannheim DNA marker X; lane 2, empty; lane 3, 41; lane 4, 43; lane 5, 44; lane 6, 66; lane 7, 67; lane 8, 68; lane 9, 69; lane 10, 42; lane 11, 70; lane 12, empty; lane 13, ATCC 20334 (*). (D) *F. sambucinum* s.str. Lane 1, Boehringer Mannheim DNA marker X; lane 2, *G. pulicaris* 76; lane 3, *F. sambucinum* 81; lane 4, 82; lane 5, 83; lane 6, 84; lane 7, 35; lane 8, 36; lane 9, 39; lane 10, 13; lane 11, 14; lane 12, 63; lane 13, 65; lane 14, empty; lane 15, ATCC 20334 (*). (E) *F. torulosum*. Lane 1, Boehringer Mannheim DNA marker X; lane 2, empty; lane 3, 85; lane 4, 86; lane 5, 87; lane 6, 88; lane 7, 89; lane 8, 90; lane 9, empty; lane 10, ATCC 20334 (*). (F) *F. venenatum*. Lane 1, Boehringer Mannheim DNA marker X; lane 2, empty; lane 3, 92; lane 4, 93; lane 5, 94; lane 6, 95; lane 7, 96; lane 8, 98; lane 9, 99; lane 10, 100; lane 11, 91; lane 12, 97; lane 13, empty; lane 14, ATCC 20334 (*).

of morphological and cultural characteristics (H. Nirenberg, pers. comm.). In addition isolates 48 and 51 were identified as *F. graminearum* (H. Nirenberg, pers. comm.). One possible explanation of their atypical RAPD banding patterns and negative PCR results with *F. graminearum*-specific primers could relate to the observations of Francis and Burgess (1977), who identified two groups of differently adapted *F. graminearum* strains (groups 1 and 2). These two groups cannot be distinguished morphologically but differ substantially with respect to epidemiological characters. It is possible that strains 48 and 51 are members of one group and that all the other *F. graminearum* strains we analyzed are members of the other group. Presumably the other three atypical strains (70, 91, and 97) could represent examples of species not included in the present study or previously undescribed species. The majority of strains representing each of the three species in question (7/9 *F. culmorum*, 9/11 *F. graminearum*, and 9/11 *F. venenatum*) originated from diverse hosts and different geographical regions yet generated identical RAPD banding patterns. Since we have already suggested (in the previous paragraph) that six strains were more similar to other species included in this analysis, we felt confident that the reason the atypical strains (70, 91, and 97) generated different banding patterns is that they are members of other species.

The results of RAPDs generated using *F. graminearum*, *F. crookwellense*, *F. sambucinum* (s.s.), *F. torulosum*, *F. venenatum*, *F. culmorum*, and ATCC 20334 DNA with primer OPW-03 are shown in Fig. 1. The inclusion of *Gibberella pulicaris* (Fries) Saccardo and *G. zeae* (Schweinitz) Petch (the teleomorphs of *F. sambucinum* s.str. and *F. graminearum*, respectively) confirmed (Figs. 1A and 1D) that the *F. sambucinum* (s.s.) and *F. graminearum* isolates

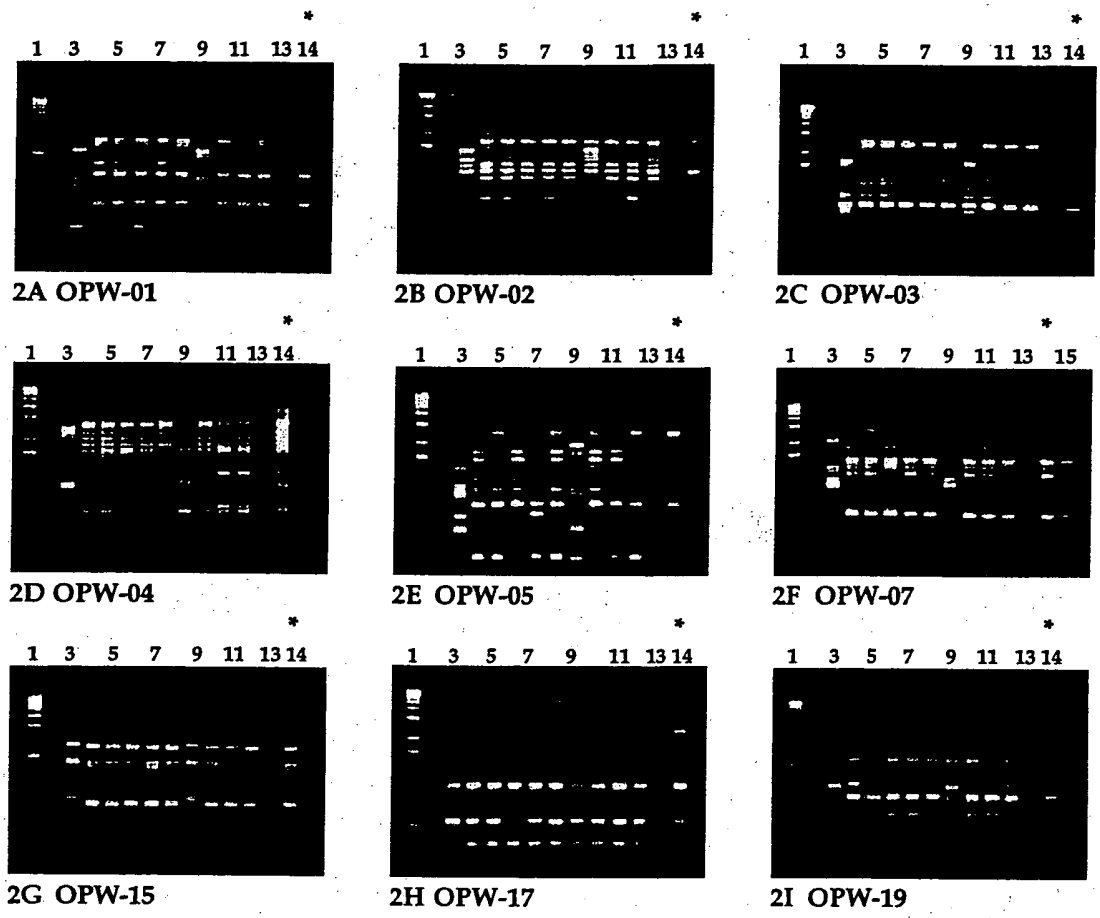


FIG. 2. RAPDs of *F. venenatum* and ATCC 20334 (*), generated using nine different primers. Lane 1, Boehringer Mannheim DNA marker X; lane 2, empty; lane 3, 91; lane 4, 92; lane 5, 93; lane 6, 94; lane 7, 95; lane 8, 96; lane 9, 97; lane 10, 98; lane 11, 99; lane 12, 100; lane 13, empty; lane 14, ATCC 20334; lane 15, 10 (3F only).

were indeed authentic. The teleomorphic states of *F. crookwellense, F. culmorum, F. torulosum,* and *F. venenatum* are not known or do not exist. RAPDs generated with 10 different primers using *F. venenatum* and ATCC 20334 as the sources of template DNA are shown in Fig. 2, and they demonstrated that with all primers ATCC 20334 gave patterns very similar to those of 9 of the 11 *F. venenatum* strains tested. As indicated above, banding patterns (and morphology on MM) of *F. venenatum* strains 91 and 97 were consistently different from those of the other *F. venenatum* strains, which may indicate that these two strains represent a closely related but distinct species/population/*formae speciales*. The current investigation did not address this issue.

It is clear that the Quorn strain is identical to the majority of the *F. venenatum* strains and unlike any of the other species tested with respect to banding pattern. However, since it is not always the case that similarly sized RAPD bands are necessarily identical in their DNA sequences, three bands (VEN-A, VEN-B, and VEN-C) from ATCC 20334 and two authentic *F. venenatum* strains (92 and 93) were cloned and sequenced. Sequence data for VEN-A, VEN-B, and VEN-C bands from these three strains (not shown) indicated 100% identity between corresponding bands. These nine sequences have been deposited with GenBank (Accession Nos. AF030562 through AF030570).

To illustrate the similarities and differences between isolates of *G. pulicaris, F. sambucinum* (s.s.), *F. torulosum,* and *F. venenatum,* RAPDs generated using primer OPW-15 and DNA from representative isolates of the above species together with ATCC 20334 were compared [Fig. 3; similar results were obtained for all other primers tested (data not shown)]. The observed banding patterns from Figs. 1 and 3 provide support for Nirenberg's contention that the three species are indeed distinct (Nirenberg, 1995).

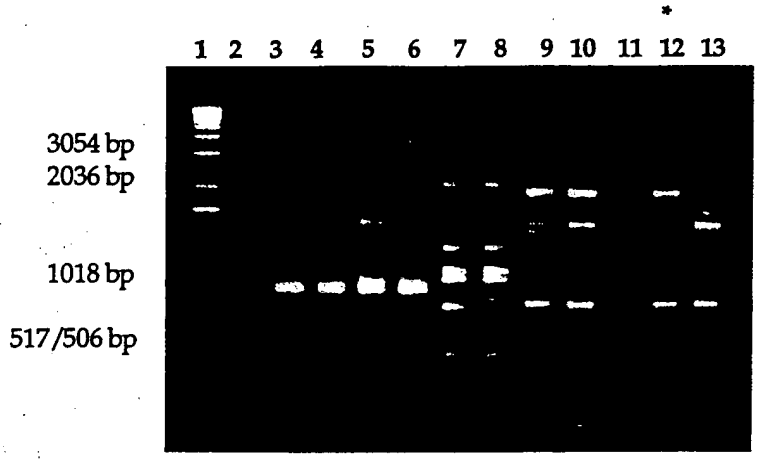


FIG. 3. Comparison of RAPDs generated from *F. sambucinum* (s.s.), *F. torulosum*, *F. venenatum*, and ATCC 20334 (*) with primer OPW-15. Lane 1, Boehringer Mannheim DNA marker X; lane 2, empty; lane 3, *G. pulicaris* 76; lane 4, *G. pulicaris* 77; lane 5, *F. sambucinum* 82; lane 6, *F. sambucinum* 83; lane 7, *F. torulosum* 87; lane 8, *F. torulosum* 89; lane 9, *F. venenatum* 92; lane 10, *F. venenatum* 93; lane 11, empty; lane 12, ATCC 20334 (*); lane 13, *F. venenatum* 10.

Species-Specific Primers

No difference was found in terms of specificity between inclusive or exclusive species-specific primers. Four pairs of RAPD-derived, species-specific primers (CRO-A, SAM-E, TOR-B, and VEN-B) amplified single bands in individual species (*F. crookwellense*, *F. sambucinum* (s.s.), *F. torulosum*, and *F. venenatum*, respectively). Two other pairs (CRO-C and CUL-A) amplified single bands in two species (*F. crookwellense* and *F. culmorum*, and *F. culmorum* and *F. graminearum*, respectively) (Fig. 4). Therefore individual primer pairs or combinations thereof could be used to differentiate unambiguously the six species included in this study. Examples of PCR products generated using species-specific primer sets are shown in Fig. 4.

When ATCC 20334 was tested with all primer sets it gave the appropriate pattern for *F. venenatum* (i.e., a band of 276 bp size only with VEN-B primers—no bands with any of the other five primers) (Fig. 4).

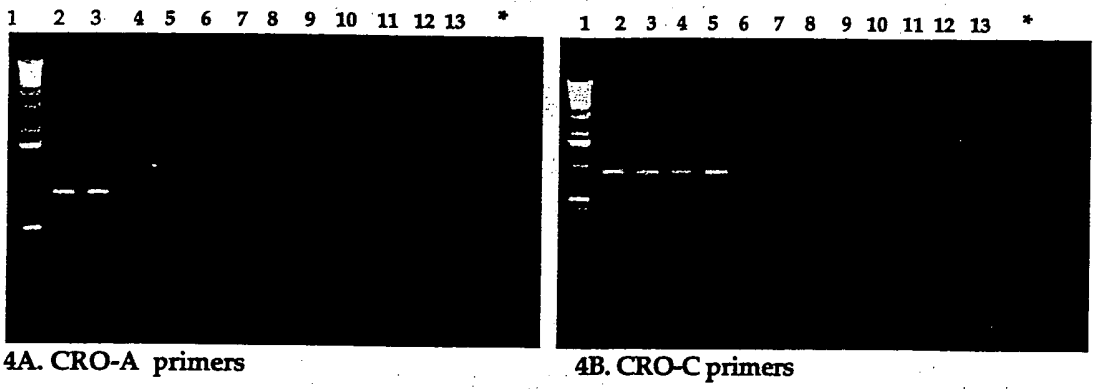
Morphological Strain Characterizations

Table 3 shows a summary of cultural and LER data on MM for all isolates studied. With the exception of those six strains mentioned above (two each of *F. culmorum*, *F. graminearum*, and *F. venenatum*), which appeared distinct from the majority of the authentic strains on the basis of morphology and RAPDs, the reference strains within species were remarkably consistent in macroscopic cultural morphology and growth rate when cultivated on MM. ATCC 20334 and *F. crookwellense* 47 are exceptional in that they are both pionnotal mutants. In addition *F. crookwellense* 47 and *F. sambucinum* 83 are slow-growing mutants, at least on the MM used in this investigation.

The difficulties in assigning some members of the genus *Fusarium* to particular species, using only morphological criteria, are evidenced by the fact that only one of the five original taxonomic opinions obtained identified ATCC 20334 as *F. venenatum* (Dr. H. Nirenberg, pers. comm.). Professor W. F. O. Marasas (pers. comm.) has also concluded (on the basis of morphology) that ATCC 20334 could be identified as *F. venenatum* according to Nirenberg (1995) or *F. sambucinum* (s.l.) according to Nelson *et al.* (1983).

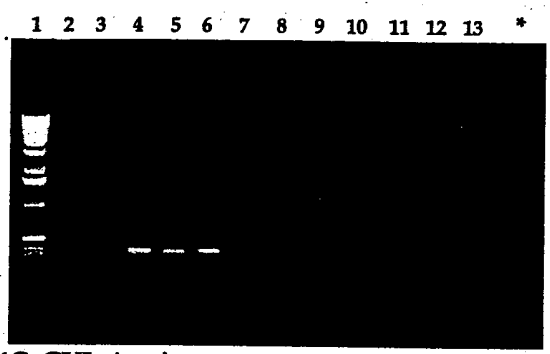
Analysis of Strains Previously Classified as *F. sambucinum* (sensu lato)

Five strains included in the present study were selected because they had previously been characterized for mating type (Desjardins and Beremand, 1987; Beremand *et al.*, 1991). The strains (61 through 65) had originally been identified as *F. sambucinum* (s.l.). However, our RAPD analyses, species-specific primer, and morphology data (Fig. 1; Table 3) suggest that only two of the strains (63 and 65) are true isolates of *F. sambucinum* (s.s.). Strain 62 appears to be a typical *F. crookwellense* on the basis of our results and the observations of J. Juba and Dr. H. Niren-

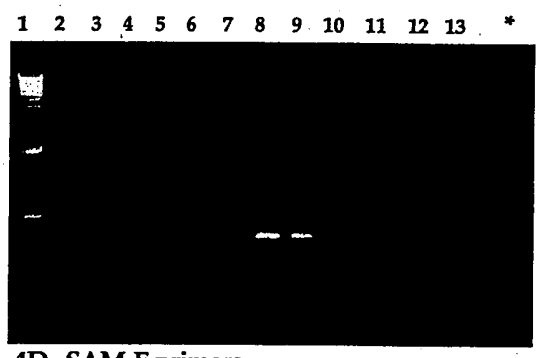


4A. CRO-A primers

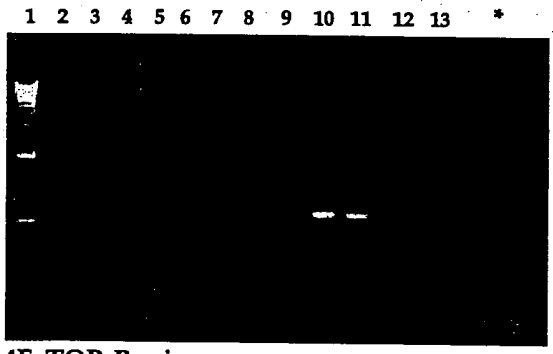
4B. CRO-C primers



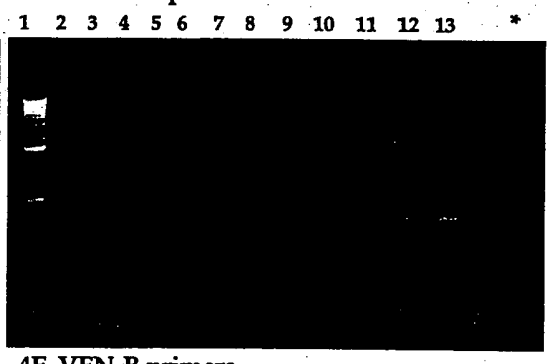
4C. CUL-A primers



4D. SAM-E primers



4E. TOR-B primers



4F. VEN-B primers

	CRO-A (842bp)	CRO-C (897bp)	CUL-A (380bp)	SAM-E (312bp)	TOR-B (664bp)	VEN-B (276bp)
F. crookwellense	+	+	-	-	-	-
F. culmorum	-	+	+	-	-	-
F. graminearum	-	-	+	-	-	-
F. sambucinum (s.s.)	-	-	-	+	-	-
F. torulosum	-	-	-	-	+	-
F. venenatum	-	-	-	-	-	+

FIG. 4. PCR products generated using species-specific primer sets and DNA from six different species in the section *Fusarium*. Lane loading (A through F): lane 1, Boehringer Mannheim DNA marker X; lanes 2 and 3, *F. crookwellense;* lanes 4 and 5, *F. culmorum;* lanes 6 and 7, *F. graminearum;* lanes 8 and 9, *F. sambucinum* (s.s.); lanes 10 and 11, *F. torulosum;* lanes 12 and 13, *F. venenatum;* lane 14, empty; * (lane 15), ATCC 20334.

TABLE 3
Cultural Morphology and Linear Extension Rates (LER) on MM

Species (no. examined)	Cultural morphology	LER (cm/day) mean (range)
F. crookwellense (14)	Abundant white/light pink/yellow floccose hyphae. Less dense at very center of colony. 7% (1/14) isolates sectored. Underside deep vinaceous red at center; white, light pink at margins. No. 47 is a slow growing morphological, pionnotal mutant with appressed orange mycelia.	0.89 (0.44 to 1.27) (including mutant 47) 0.92 (0.68 to 1.27) (excluding 47)
F. culmorum (7)	White to whitish-yellow mycelia, more appressed in older regions of colony. Sparse, whitish-yellow strands of aerial mycelia at margins. 14% (1/7) isolates sectored. Underside vinaceous red (patchy in some isolates).	0.86 (0.79 to 0.97)
No. 42	White, floccose aerial mycelia. Underside very light vinaceous red in center, white at margins.	0.86
No. 70	Older regions pink, appressed. Floccose white mycelia at margins. Sectoring evident in this isolate. Underside very light pink.	0.71
F. graminearum (9) *G. zeae* (1)	Appressed, dense mycelia (velvety in some isolates), sparsely floccose. Hyphae becoming sparse toward colony margin. Underside pink to deep vinaceous red in older regions becoming light pink to white at the colony margin.	0.67 (0.58 to 0.86)
No. 48	Dense aerial hyphae from deep pink in older regions to pink-white to greenish-yellow-brown. Underside deep vinaceous red.	0.39
No. 51	Appressed pink mycelia at center of colony, becoming densely aerial, yellow to pinkish-white at margins. Underside intense vinaceous red except at growing tips, which are white.	0.56
F. sambucinum (11) *G. pulicaris* (2)	Salmon pink, orange, to yellowish-green farinaceous, feathery, appressed mycelia. In some strains growing tips white. One strain sparse, pink-white floccose mycelia. 7.6% (1/13) sectored. Underside same color as aerial hyphae.	0.64 (0.21 to 0.79) (including mutant strain 83) 0.67 (0.54 to 0.79) (excluding 83)
F. venenatum (11)	Older parts of colony appressed, with varying degrees of more pronounced aerial mycelium at margins. Hyphae yellow-brown to salmon pink to white. 9% (1/11) with very pronounced sectoring. Underside bright yellow, brown, salmon pink-orange to red. ATCC 20334 is a pionnotal mutant with somewhat reduced aerial mycelium.	0.71 (0.45 to 0.82)
Nos. 91 and 97 (2)	White-cream, dense, cottony, appressed mycelia. Underside very faint orange-yellow.	0.634 (0.54 to 0.73)
F. torulosum (7)	Dense, velvety, felt-like mycelia, white to light creamy-yellow. 16% (1/7) sectored. Underside deep rose red-pink, but with white margins.	0.18 (0.15 to 0.21)

berg (pers. comm.), while strains 61 and 64 appear to be *F. torulosum* and *F. venenatum*, respectively, on the basis of our data and the opinion of Dr. H. Nirenberg (pers. comm.). The proposed identities of these and other isolates from the section *Fusarium* will be the subject of a subsequent publication and will not be discussed in further detail here.

DISCUSSION

Identification of *Fusarium* species based on morphological and cultural criteria is not always straightforward, even for expert taxonomists. The genus *Fusarium* is an assemblage of mitosporic fungi grouped together on the basis of morphological criteria which can be highly variable depending on media and culture conditions. Thus, using current keys, genetically unrelated strains could potentially be artificially classified as the same species. Given this situation plus the fact that several different taxonomic systems exist, reflected in the taxonomic treatises of, for example, Snyder and Hansen (1945), Nelson *et al.* (1983), Booth (1971 and 1977), Burgess *et al.* (1988), and Gerlach and Nirenberg (1982), it is perhaps not suprising that four different opinions concerning the identity of ATCC 20334 were rendered by the various independent authorities we consulted. We therefore attempted to devise a rigorous taxonomic method based on molecular and morphological criteria for distinguishing, reliably, the six species included in this study, which are currently grouped in the section *Fusarium*.

A relatively high percentage [18% (12/67)] of the "authentic" strains appeared to have been previously misidentified. This situation made it difficult to establish a baseline from which to identify species-specific differences. However, since many isolates of worldwide distribution were examined it was possible to identify the common species-specific RAPD banding patterns generated by the majority of isolates of each species. The atypical *F. culmorum* (70) and *F. venenatum* (91 and 97) isolates may possibly represent species not included in the present investigation. Similar numbers of misidentified *Fusarium* isolates were reported in a study by Niessen and Vogel (1997). These authors used a PCR-based method to specifically identify *F. graminearum* isolates by using a primer pair based on the sequence of the galactose oxidase (*gao*A) gene, which is specific for *F. graminearum, F. fujikuroi* Nirenberg, and *Beltraniella portoricencis* (F. Stevens) Piroz and S. D. Patil, in conjunction with morphological and enzymatic criteria. They found that of 22 isolates originally identified as *F. graminearum*, 4 (18%) were other, closely related species. Marasas *et al.* (1984) also describe a multitude of misidentified toxigenic *Fusarium* strains. Increased application of the appropriate molecular methods in conjunction with complementary morphological and other criteria would undoubtedly help clarify *Fusarium* taxonomy.

Although the number of strains of each species used in this investigation was small, the intraspecific RAPD banding patterns generated from them were remarkably consistent, considering their diverse origins. The uniformity observed among strains belonging to the same species suggests that there is not a major mechanism for generating variation in these species, although it is possible that parts of the genome, not sampled by the random primers we used, are more variable. Interestingly, the highest degree of variation was observed between isolates of *F. sambucinum* (s.s.), which is heterothallic and thus would be expected to be more variable than suspected nonsexual species, such as *F. crookwellense*, or homothallic species, such as *F. graminearum/G. zeae*. Ouellet and Seifert (1993) found little genetic diversity among the 19 *F. graminearum* strains they tested using RAPDs and restriction analysis of PCR fragments. Also, Bateman *et al.* (1996) found little diversity among strains of the same species by comparing RFLPs in PCR-amplified nuclear rDNA from 35 species of *Fusarium* from 12 different sections.

Nirenberg (1995) provided convincing evidence for the morphological differentiation of *F. sambucinum* (s.l.) into three species, *F. sambucinum* (s.s.), *F. torulosum*, and *F. venenatum*. Further support for this differentiation was provided by Hering and Nirenberg (1995), who employed RAPDs to differentiate *F. sambucinum* (s.l.) and related species, although these authors used a minimal number of primers and provided no evidence of sequence similarity between similarly sized bands. Logrieco *et al.* (1995) provided more molecular support for the morphological interpretation distinguishing the three taxa, based on comparisons of ribosomal RNA sequences. O'Donnell and Cigelnik [unpublished observations reported in O'Donnell and Cigelnik (1997)] found that phylogenies generated from four data sets indicated that these three cryptic species are distinct phylogenetically. The three taxa have also been distinguished on the basis of their secondary metabolite profiles (Thrane and Hansen, 1995; Altomare *et al.*, 1995) and their mating behavior (Desjardins and Nelson, 1995). Our RAPD data and morphological characterizations also support Nirenberg's proposed division.

RAPD banding patterns (generated using 10 different random decamer primers), species-specific primer PCR results, and sequence analyses of three similarly sized bands from ATCC 20334 and two reliably identified strains of *F. venenatum*, one of which was the ex-type culture, shared little or no interstrain variation. These molecular results, combined with our observations of colony characters, strongly support the identification of ATCC 20334 as *F. venenatum*.

Two published observations can now be clarified if ATCC 20334 is indeed *F. venenatum*. Bu'lock *et al.* (1986) reported that the method of producing forced heterokaryons by adjacent inoculation onto minimal agar was not effective for generating heterokaryons between derivatives of A3/5 (= ATCC 20334) and a high zearelenone-producing *F. graminearum* strain (NRRL 3198), while the technique worked successfully when both the parent strains were derived from NRRL 3198. Not surprisingly, these authors were able to obtain heterokaryons of the former type via protoplast fusion. Ouellet and Seifert (1993) found DAOM 212262 [= A3/5 = ATCC 20334 (A. P. J. Trinci, pers. comm.)] was very different from other *F. graminearum* isolates examined with respect to RAPDs and restriction analysis of PCR-amplified fragments, an observation that could also be explained on the basis of our proposed reclassification of ATCC 20334 as *F. venenatum*.

With respect to the general safety of Quorn as an edible product, no negative implications are to be expected as a result of the proposed species designation we are recommending for ATCC 20334. Thorough animal (e.g., Duthie, 1975), human (e.g., Trinci, 1992), bacteriological (e.g.,

Solomons, 1986), and plant pathogenicity (e.g., U.S. patent 4,501,765) safety tests which this strain has undergone over the past 20 years were conducted on strain A3/5 (= ATCC 20334), regardless of what the strain's specific epithet may have been at any time during this period.

The issue of phylogenetic relationships among the species included in this study was not our major focus and has therefore not been addressed. However, our observation that species-specific primer sets CRO-C and CUL-A do not distinguish *F. crookwellense/F. culmorum* and *F. culmorum/F. graminearum*, respectively, support the recent findings of Mule *et al.* (1997). In their analysis *F. crookwellense*, *F. culmorum*, and *F. graminearum* were found to be more closely related to one another than to *F. sambucinum*, *F. venenatum*, and *F. tumidum* Sherb. on the basis of 28S rDNA sequence comparisons.

Concerning the general use of species-specific primers to identify unknown strains, it is conceivable that false negative results could be obtained. If no bands are generated under conditions that amplify bands from the positive control and that would be expected to generate bands in an unknown isolate (due to morphological or other considerations), then comparative RAPDs should be run with authentic isolates of the species with which the unknown *Fusarium* strain is suspected of having affinity. It is possible that single base pair mutations in regions homologous to the primers could reduce the homology sufficiently to inhibit annealing and thus result in no bands of the expected size being generated. Development and use of two independent species-specific primer pairs per species would be expected to significantly reduce the chances of such false negatives occuring.

The design of RAPD-derived, species-specific primers and their successful application in distinguishing members of different *Fusarium* species reinforces the results of other investigators (e.g., Niessen and Vogel, 1997; Voigt *et al.*, 1995). Hopefully these types of investigation will serve to underline the importance of applying combinations of (at least two) different approaches (e.g., RAPDs/PCR using species-specific primers; sequence comparisons of multiple, appropriate genes; secondary metabolite profiles; isozyme analysis; and cultural and morphological criteria) to establish baselines for the identity of mitosporic species representing genera which are problematic to taxonomists, such as *Fusarium*, *Aspergillus*, and *Penicillium*. The development and application of RAPD-derived, species-specific primers for distinguishing *Fusarium* species in this and other studies (e.g., Schilling, 1996) suggest that this technique may find widespread use as a diagnostic tool in the genus *Fusarium*. Since 19 of the first 20 random primers tested by us on 67 isolates belonging to this genus generated clear, characteristic, species-specific RAPD banding patterns, it seems likely that other sections may also be amenable to this type of analysis. After development and subsequent verification with a specified number of "authentic" strains, sequences of species-specific primers could conceivably be deposited in GenBank. Perhaps "FusKits," containing primers to resolve all known members of the different sections, will ultimately become available and the utilization of species-specific primers for identification of unknown isolates by nonexpert taxonomists will become routine. The benefits of such a simple yet precise diagnostic tool to plant pathologists, animal toxicologists, taxonomists, and phylogenetecists would be substantial.

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Molecular Phylogenetic, Morphological, and Mycotoxin Data Support Reidentification of the Quorn Mycoprotein Fungus as *Fusarium venenatum*

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O'Donnell, K., Cigelnik, E., and Casper, H. H. 1998. Molecular Phylogenetic, Morphological, and Mycotoxin Data Support Reidentification of the Quorn Mycoprotein Fungus as *Fusarium venenatum*. *Fungal Genetics and Biology* **23,** 57–67. Molecular phylogenetic, morphological, and mycotoxin data were obtained in order to investigate the relationships and identity of the Quorn mycoprotein fungus within *Fusarium* and to examine Quorn strains and commercial Quorn food products for trichothecene mycotoxins. Phylogenetic analyses of aligned DNA sequences obtained via the polymerase chain reaction from the nuclear 28S ribosomal DNA, nuclear ribosomal internal transcribed spacer region, and β-tubulin gene exons and introns indicate that the Quorn fungus is *Fusarium venenatum*, rather than *F. graminearum* as previously reported. All of the Quorn strains examined were morphologically degenerate aconidial colonial mutants except for NRRL 25139, which produced chlamydospores in recurved terminal chains together with mostly 5-septate sporodochial conidia on doliform monophialides diagnostic of *F. venenatum*. Bootstrap and decay analyses provide strong support for a monophyletic lineage containing *F. venenatum* and several other type A trichothecene-producing species, while reference strains of *F. graminearum* were nested in a separate clade of species that produce type B trichothecenes and/or zearalenone. Analysis of mycotoxins from rice cultures inoculated with Quorn strain NRRL 25416 revealed that four type A trichothecenes are produced, but at low levels relative to strain NRRL 22198 of *F. venenatum*. No trichothecene mycotoxins, however, were detected from the analysis of three commercial Quorn products marketed for human consumption in England. © 1998 Academic Press

Index Descriptors: Fusarium; mycoprotein; mycotoxins; phylogenetics; Quorn; trichothecenes.

A filamentous fungus identified as *Fusarium graminearum* Schwabe A 3/5 has been cultured as a mycoprotein source for human consumption in England for over a decade under the registered trade name Quorn (Trinci, 1992, 1994a,b). Heightened interest in this fungus stems from its recent use as a host for the expression of heterologous proteins for use in a wide range of industrial applications (Royer *et al.*, 1995). Although Quorn strain A 3/5 has been reported to produce no toxic secondary metabolites (Solomons, 1986), many fusaria are noted for a plethora of mycotoxins, including *F. graminearum*, which can produce zearalenone and type B trichothecenes such as deoxynivalenol (Marasas *et al.*, 1984). Zearalenone is an estrogenic metabolite, whereas trichothecenes pose a threat to plant and animal health, including humans, because they can inhibit protein synthesis (Marasas *et al.*, 1984; Desjardins *et al.*, 1993).

Accurate identification of *Fusarium* species is problematical for both specialists and nonspecialists alike given the paucity and plasticity of morphological characters combined with discordant and often polytypic morphological

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species concepts employed in the three most widely used taxonomic treatments of the genus (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983). Identification of atypical or degenerate cultures, such as the aconidial colonial mutants of Quorn strain A 3/5 (Wiebe *et al.*, 1991), is often impossible using morphology alone. Fortunately, molecular phylogenetics of discrete DNA sequence data provides an avenue through which species limits can be investigated, thereby providing a means by which any isolate of *Fusarium* can be identified objectively independent of cultural morphology (Guadet *et al.*, 1989; O'Donnell, 1996).

In the present study, nucleotide data from three regions were obtained by sequencing DNA amplified via PCR in order to investigate the phylogenetic relationships and identity of the Quorn mycoprotein strain previously identified as *F. graminearum* A 3/5 (Trinci, 1994b). In addition to the parent Quorn strain A 3/5, several colonial mutants were examined morphologically using a protocol outlined by Nirenberg (1995) for the identification of *F. venenatum* Nirenberg. Last, two Quorn strains and three commercial Quorn products from England were screened for trichothecene mycotoxins in order to evaluate the potential health hazard from human consumption of this mycoprotein.

MATERIALS AND METHODS

One wild-type Quorn strain (NRRL 26139) and six aconidial colonial mutant Quorn strains (NRRL 25412-25417) received as *F. graminearum* (Table 1), and three commercial Quorn mycoprotein products, were compared with two reference strains of *F. graminearum* together with phenotypically similar species within the morphology-based infrageneric sections *Discolor* and *Sporotrichiella* (Gerlach and Nirenberg, 1982). Two or more reference strains were used for all of the described species included in this study, except for *F. lunulosporum* and *F. robustum* for which the ex-type strain of each was analyzed. Inclusion of the ex-type strain of *F. venenatum* (NRRL 26139 = CBS 458.93 = BBA 64537) provided the basis for a direct comparison of the Quorn mycoprotein fungus with this species (see below).

All strains are stored by lyophilization or cryogenically at −175°C in the Agriculture Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research (Peoria, IL) (Table 1). Mycelium for DNA extraction was grown in yeast–malt broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% dextrose; Difco; Detriot, MI), harvested by vacuum filtration, and freeze-dried as described previously (O'Donnell, 1992). A hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO) miniprep protocol (O'Donnell *et al.*, 1997) was employed for extraction of total genomic DNA from approximately 50 mg of lyophilized mycelium.

Dilute DNA templates for PCR were prepared by adding 4 μl of the total genomic DNA stocks to 1 ml of double-distilled H_2O. Sequences and locations of the polymerase chain reaction and sequencing primers have been published (White *et al.*, 1990; O'Donnell, 1996; O'Donnell and Cigelnik, 1997). All PCR amplifications were conducted with a Perkin–Elmer 9600 thermal cycler using the fastest ramp times as follows: 40 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, followed by a 4°C soak. Amplified DNA was purified with GeneClean II (Bio 101, La Jolla, CA) and sequenced with the Applied Biosystems Dye Terminator Cycle Sequencing kit (Foster City, CA) in a Perkin–Elmer 9600 thermal cycler employing the fastest ramp times as follows: 25 cycles of 15 s at 96°C and 4 min at 55°C, followed by a 4°C soak. All sequencing reaction mixtures were analyzed with an Applied Biosystems 373A DNA sequencer. Sequences have been deposited with GenBank (see Table 1 for accession numbers) and are also available from the authors in the aligned format upon request.

Following visual alignment of the DNA sequences with TSE, a DOS text editor software program (SemWare, Marietta, GA), maximum parsimony trees were inferred from the separate and combined dataset with PAUP*4.0d54 (Swofford, 1993, 1997). Heuristic searches consisted of 1000 random addition sequences, rooting trees by the outgroup method. Clade stability was assessed with 1000 bootstrap replications (Hillis and Bull, 1993) and by decay indices calculated with TreeRot (Sorenson, 1996). Base pair composition and the transition/transversion pattern of the combined dataset were estimated with MacClade (Maddison and Maddison, 1992). A phylogenetic species concept was adopted in this study in which species are defined as exclusive, independently evolving lineages which are diagnosed by a unique combination of fixed, derived characters (i.e., apomorphies).

For morphological observations, strains were cultivated on modified synthetic nutrient agar (SNA)[1] (per liter: 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g dextrose, 0.2 g sucrose, 0.6 ml 1 N NaOH, 23 g agar) in

[1] Abbreviations used: ITS, internal transcribed spacer; SNA, synthetic nutrient agar; EI, electron ionization; TMS, trimethylsilyl; SIM, select ion monitoring; SCIRP, scirpentriol; 15AC-SCIRP, 15-acetoxyscirpenol; DAS, diacetoxyscirpenol; 4-MAS, 4-monoacetoxyscirpenol.

TABLE 1

Strains Sequenced and Analyzed Phylogenetically

Species	NRRL strain no.	Equivalent no.[a]	GenBank accession nos.[b]		
			28S	ITS	β-Tubulin
F. cerealis Cooke[c]	13721	KF-748	U85517	U85534	U85568
F. cerealis	25491	CBS 589.93	AF006320	AF006340	AF006360
F. cerealis	25805	CBS 195.80	AF006321	AF006341	AF006361
F. culmorum (W. G. Smith) Sacc.	3288	Prentice 3737	U85518	U85535	U85569
F. culmorum	25475	CBS 417.86	AF006322	AF006342	AF006362
F. flocciferum Corda	13383	FRC R-5204	U85519	U85536	U85570
F. flocciferum	25471	CBS 792.70	AF006323	AF006343	AF006363
F. graminearum Schwabe	5883	GZ-17	U34549	U34578	U34436
F. graminearum	25797	CBS 415.86	AF006324	AF006344	AF006364
F. lunulosporum Gerlach	13393	FRC R-5822	U85520	U85537	U85571
F. oxysporum Schlecht.	22902	BCRI 9065I	U34537	U34566	U34424
F. poae (Peck) Wollenw.	13714	FRC T-503	U85521	U85538	U85572
F. poae	25799	CBS 446.67	AF006325	AF006345	AF006365
F. robustum Gerlach	13392	FRC R-5821	U85522	U85539	U85573
F. sambucinum Fuckel	22187	BBA 64226	U85523	U85540	U85574
F. sambucinum	22203	BBA 62719	AF006326	AF006346	AF006366
F. sporotrichioides Sherb.	13440	FRC T-521	U85524	U85541	U85575
F. sporotrichioides	25474	CBS 448.67	AF006327	AF006347	AF006367
F. sporotrichioides	25479	CBS 447.67	AF006328	AF006348	AF006368
F. tumidum Sherb.	13394	FRC R-5823	U85525	U85542	U85576
F. tumidum	22240	CBS 486.76	AF006329	AF006349	AF006369
F. venenatum Nirenberg	22196	BBA 65031	U85526	U85543	U85577
F. venenatum	22198	BBA 64478	AF006330	AF006350	AF006370
F. venenatum[d]	25412	IMI 154213	AF006331	AF006351	AF006371
F. venenatum[d]	25413	IMI 154212	AF006332	AF006352	AF006372
F. venenatum[d]	25414	IMI 154211	AF006333	AF006353	AF006373
F. venenatum[d]	25415	IMI 154210	AF006334	AF006354	AF006374
F. venenatum[d]	25416	IMI 154209	AF006335	AF006355	AF006375
F. venenatum[d]	25417	IMI 145425	AF006336	AF006356	AF006376
F. venenatum[d]	26139	ATCC 20334	AF006337	AF006357	AF006377
F. venenatum[e]	26228	CBS 458.93	AF006338	AF006358	AF006378
F. venenatum[f]	—	—	AF006339	AF006359	AF006379
Fusarium sp.	22189	BBA 64262	U85531	U85548	U85582
Fusarium sp.	22192	BBA 64918	U85532	U85549	U85583

Quorn

[a] BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany; BCRI, British Columbia Research Inc., Vancouver, Canada; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; FRC, Fusarium Research Center, Department of Plant Pathology, Pennsylvania State University, University Park, PA; GZ-17, NRRL isolate *Gibberella zeae* (Schweinitz) Petch; KF, Department of Plant Pathology, Argicultural University of Warsaw, Pozan, Poland; IMI, CAB International Mycological Institute, Egham, UK; Prentice, N. Prentice, ARS, Madison, WI from W. C. Gordon, Winnipeg, Canada.

[b] http://www.ncbi.nlm.nih.gov.

[c] *F. cerealis* [syn = *F. crookwellense* Burgess *et al.* (1982)].

[d] Quorn mycoprotein strain.

[e] Ex-type strain of *F. venenatum* received as CBS 458.93 (= BBA 64537).

[f] Received as Quorn mycoprotein pieces; Marlow Foods Limited, Stokesley, Cleveland TS9 7AB, England.

100 × 15-mm plastic petri dishes with a 1 × 2-cm piece of sterile filter paper placed on the cooled agar surface (Nirenberg, 1995). Cultures were incubated at 25°C for 3 weeks either in complete darkness or under continuous black light (Philips TLD 18 W/08). Micromorphological features were viewed, measured, and photographed with a Zeiss Axioplan microscope equipped with differential interference optics (Table 2). Length and width differences were examined by standard analysis of variance.

Strains were tested for their ability to produce trichothecene mycotoxins following culture on rice grain. Erlenmeyer flasks (500 ml) with Morton closures were

TABLE 2

Comparison of Key Morphological Features of *Fusarium venenatum* Quorn Strain NRRL 26139 with the Ex-Type Strain NRRL 26228 and *F. graminearum*

		5-septate macroconidia		
	Phialides	Black light (SD)	Complete darkness (SD)	Chlamydospores
F. venenatum[a] [Quorn strain]	Mostly doliform	39.5–55.5 × 5.2–6.7 µm $\bar{x}$ 47.4 (3.4) × 6.0 (0.4) µm	37.5–49.5 × 4.8–6.7 µm $\bar{x}$ 43.3 (3.5) × 5.9 (0.4) µm	Abundant, terminal recurved chain
F. venenatum [ex-type strain]	Mostly doliform	37.5–54.0 × 4.8–6.7 µm $\bar{x}$ 45.7 (3.0) × 5.9 (0.3) µm	36.0–48.0 × 5.7–6.7 µm $\bar{x}$ 41.3 (2.8) × 6.0 (0.3) µm	Abundant, terminal recurved chain
F. graminearum[b]	Mostly cylindric to subcylindric	28–72 × 3.2–6.0 µm $\bar{x}$ 51 × 4.9 µm		Scarce, mostly intercalary

[a] Measurements of 5-septate macroconidia (n = 100) of *F. venenatum* Quorn strain NRRL 26139 and the ex-type strain NRRL 26228 were from cultures grown under continuous black light or in complete darkness as described under Materials and Methods. SD, standard deviation.
[b] Measurements from Gerlach and Nirenberg (1982).

filled with 50 g of long grain converted rice (Uncle Ben's, Houston, TX) and 100 ml of sterile distilled water and were allowed to soak for 6 h prior to autoclaving for 30 min. Once the flasks had cooled to room temperature, each was inoculated with 5 ml of a suspension of mycelium taken from 4-day-old yeast–malt broth cultures of the strains tested (Table 2). Strain NRRL 22198 (= BBA 64478) of *F. venenatum*, a known producer of the type A trichothecene diacetoxyscirpenol (DAS; Altomare *et al.*, 1995; Schmidt *et al.*, 1995; Thrane and Hansen, 1995), was used as a positive control. The negative control consisted of a rice culture inoculated with 5 ml of sterile distilled water. Each flask was shaken once daily for the first 3 days to break up clumps of rice and to distribute the inoculum. Once inoculated, the flask cultures were incubated in complete darkness at 25°C for 30 days at which time they were freeze-dried for 2 days, ground to a fine powder in a laboratory mill, and stored at −20°C until analyzed.

Fusarium mycotoxins were extracted from 5 g of ground culture material with 20 ml acetonitrile:water (84 + 16) for 1 h on a horizontal shaker. A 6-ml aliquot of the supernatant was gravity filtered through 1.5 g of C18:Alumina

TABLE 3

Trichothecenes Detected in Rice Cultures Inoculated with *Fusarium venenatum* and in Three Commercial Quorn Mycoprotein Products Sold in the United Kingdom (Marlow Foods Limited, Stokesley, Cleveland, England)

	Concentration (µg/g dry weight basis)[a]			
Strain or product	SCIRP[b]	15AC-SCIRP[c]	DAS[d]	4-MAS[e]
NRRL 22198	567	291	911	339
NRRL 25416	45	8	32	34
NRRL 26139	0	0	0	0
Quorn pieces	0	0	0	0
Quorn mince	0	0	0	0
Quorn quarter pounders	0	0	0	0

[a] Estimated detection limit is 0.5 µg/g.
[b] SCIRP, scirpentriol.
[c] 15AC-SCIRP, 15-acetoxyscirpenol.
[d] DAS, diacetoxyscirpenol.
[e] 4-MAS, 4-monoacetoxyscirpenol.

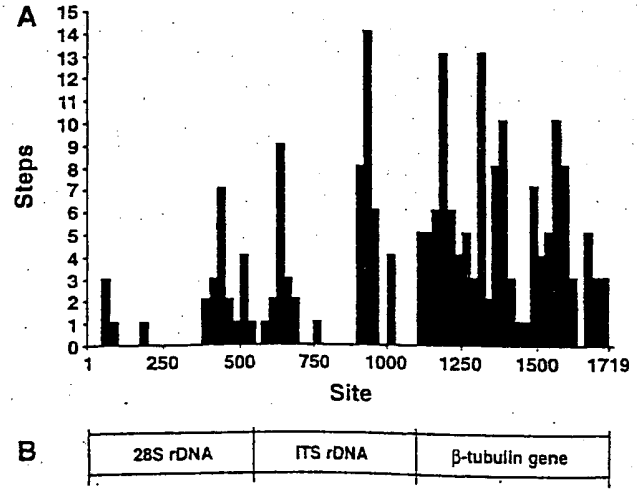


FIG. 1. (A) MacClade chart of the 24 equally most-parsimonious trees found by PAUP, using a 25-bp interval, showing the distribution of steps (= substitutions) within each of the three regions sequenced that constitute the combined dataset. Sequences of *F. oxysporum* were used as an alternate, more distant outgroup to root the trees (see phylogram in Fig. 5), but they were not included in the MacClade analysis. (B) Map of the three regions sequenced and analyzed phylogenetically (28S rDNA, 535 bp; ITS rDNA, 562 bp; β-tubulin gene, 662 bp).

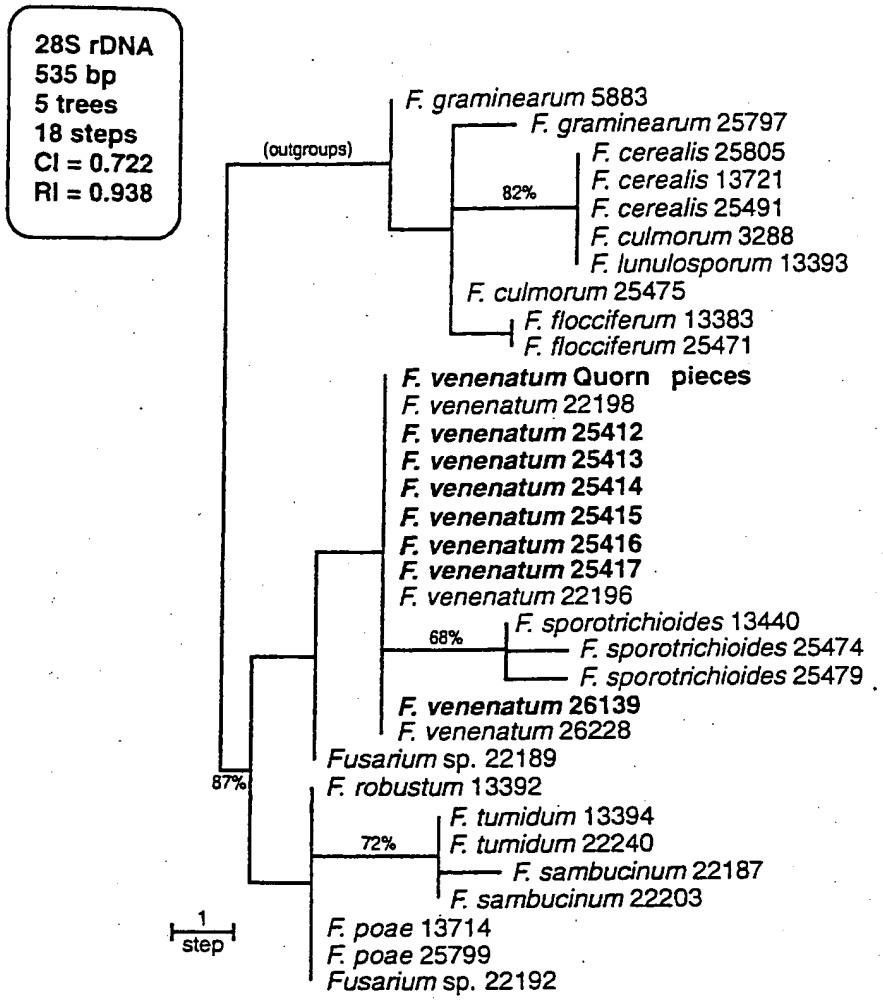


FIG. 2. One of five equally most-parsimonious phylograms for the nuclear 28S rDNA sequences rooted by the outgroup method. Bootstrap replication frequencies above 50% and edge length (steps) are indicated. CI, consistency index; RI, retention index. Quorn and Quorn strains are in bold type.

(1 + 1), and a 2-ml aliquot of the eluent was evaporated with nitrogen at 55°C for 30 min. The residue was derivatized (*N*-trimethylsilyimidazole + trimethylchlorosilane + *n,o*- bis[trimethylsily]trifluoroacetamide + pyridine) to form trimethylsilyl (TMS) ester derivatives of trichothecenes and estrogens. The TMS–mycotoxin derivatives were separated on a Hewlett–Packard gas chromatograph, using a Restek 30-m RTX 35 × 0.25-mm-i.d. × 0.25-μm-phase capillary column, and assayed by select ion monitoring (SIM) with electron ionization (EI) in a Finnigan Incos 50 mass spectrometer, using 3 or 4 ion fragments for identification and quantitation of each mycotoxin. The capillary column was held at 110°C for 6 s after on-column injection, heated to 210°C at 25°C/min, and then heated to 300°C at 5°C/min and held for 5 min (27.1 min total) before recycling. Helium linear velocity at 110°C was 35 cm/s. Mycotoxins that can be identified in this manner are nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, diacetoxyscirpenol, scirpentriol, 15-acetoxyscirpentriol, T-2 toxin, iso-T-2 toxin, acetyl-T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, neosolaniol, zearalenol, and nearalenone (Savard and Blackwell, 1994). General recoveries of mycotoxin at 1 ppm range from 80 to 100%. The estimated detection limit for the above mycotoxins, using SIM, is 0.5 μg/g.

After the preliminary runs in SIM, it was obvious that none of the above mycotoxins were present in the uninoculated negative control, three commercial Quorn mycoprotein products (pieces, mince, and quarter pounders; Marlow Foods Limited, Stokesley, Cleveland, England), and

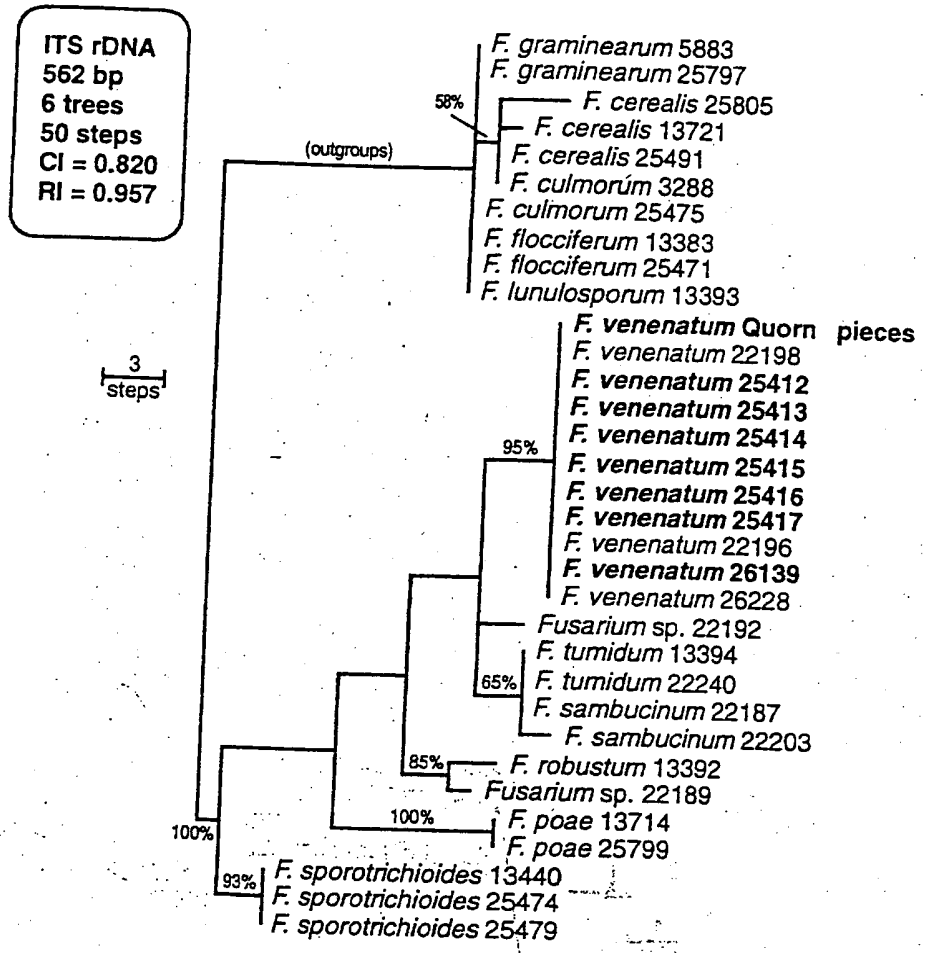


FIG. 3. One of six equally most-parsimonious outgroup rooted trees for the nuclear rDNA ITS sequences. Edge length and bootstrap percentages above 50% are indicated. CI, consistency index; RI, retention index. Quorn and Quorn strains are in bold type.

the wild-type Quorn strain NRRL 26139. However, rice cultures inoculated with NRRL 22198 *F. venenatum* and Quorn strain NRRL 25416 *F. venenatum* contained high levels of several toxins, and a full scan program (50–650 *m/z* per second) was used to confirm their identity and to estimate their concentration. The full scan identified the presence of scirpentriol (SCIRP), 15-acetoxyscirpenol (15AC-SCIRP), DAS, and 4-monoaceytoxyscirpenol (4-MAS). Pure standards from Sigma Chemical Co. (St. Louis, MO) were used for the identification and quantitation of SCIRP, 15AC-SCIRP, and DAS. Because the 4-MAS standard provided by Dr. George Rottinghaus (University of Missouri, Columbia) did not have quantitative status, the 4-MAS concentration was based upon its total ion response compared to the DAS standard. Identification of the four mycotoxins was based upon the EI spectra and retention times compared to known standards (Table 3).

RESULTS

Three regions (Figs. 1A and 1B) were sequenced and analyzed phylogenetically for all 33 strains listed in Table 1, including commercial Quorn mycoprotein pieces, mince, and quarter pounders. DNA sequences obtained from mince and quarter pounders were identical to those from pieces (data not shown). The β-tubulin gene copy we sequenced is orthologous with *tub*2 of *F. verticillioides* (Sacc.) Nirenberg (syn. = *F. moniliforme* Sheldon; Yan and Dickman, 1996) and *ben*A of *Aspergillus nidulans* (Eidam) Wint. (May *et al.*, 1987). Sequences of the β-tubulin gene contributed 69.3% of the parsimony-informative characters in the combined dataset, followed by the nuclear internal transcribed spacer (ITS) rDNA region (21.8%) and the 5′-most portion of the nuclear 28S rDNA (8.9%).

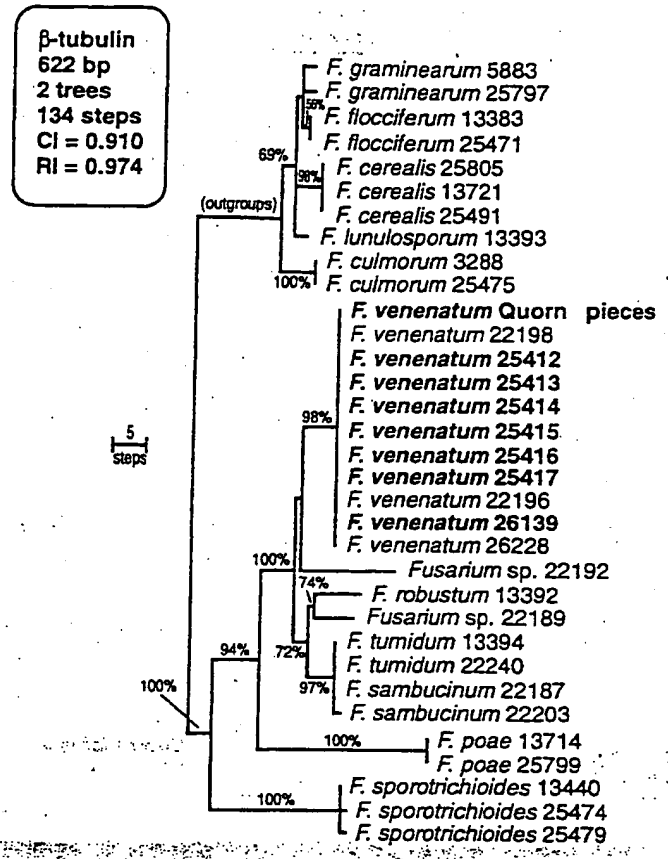


FIG. 4. One of two equally most-parsimonious phylograms inferred from the β-tubulin gene sequences rooted by the outgroup method. Bootstrap intervals above 50% and edge length are indicated. CI, consistency index; RI, retention index. Quorn and Quorn strains are in bold type.

The average transition/transversion ratio approximated with MacClade for the 24 equally most-parsimonious trees found with PAUP for the combined dataset is 3.0. Substitutional mutation values were tested with χ^2 goodness of fit techniques. A significant ($P < 0.01$) bias toward transitions was detected (108 of 144 unambiguous substitutions = 75%, for tree 1 of 24), and among transitions, substitutions were strongly biased toward C ⇌ T mutations ($P < 0.01$; 76 of 108 unambiguous transitions = 70.3%). The central portion of the nuclear 28S and ITS (= 5.8S rDNA) rDNA sequences (Fig. 1A) contains highly conserved regions. Published sequences (O'Donnell, 1992) were used to identify spacer-genic boundaries within the ITS region.

Phylogenetic analysis of sequences from the 5′-most portion of the nuclear 28S rDNA yielded five largely unresolved, equally most-parsimonious trees in which the outgroup species contributed 8 of the 18 steps (Fig. 2). Reference strains of *F. graminearum* clustered with the outgroup species within the 28S rDNA tree, whereas the Quorn mycoprotein strains grouped with strains of *F. venenatum*, including the ex-type culture NRRL 26228 (= CBS 458.93). Maximum parsimony analysis of the rDNA ITS region (Fig. 3), β-tubulin gene introns and exons (Fig. 4), and the combined dataset (Fig. 5) revealed considerable phylogenetic structure within the ingroup, which was strongly supported as monophyletic (100% bootstrap). A heuristic search implemented by PAUP, using 1000 random addition sequences, yielded 6, 2, and 24 equally most-parsimonious outgroup-rooted trees for the ITS region, β-tubulin gene, and combined data (Figs. 3–5), respectively. Gene trees inferred from β-tubulin (Fig. 4) and the combined data (Fig. 5) identified *F. sporotrichioides* (section *Sporotrichiella* Wollenw.) as a sister of the remaining ingroup taxa. Within this latter clade, *F. poae* (Peck) Wollenw. (section *Sporotrichiella*) formed a sister to a strongly supported clade containing the Quorn mycoprotein strains and *F. venenatum* (98–100% bootstrap; decay index = 9; Figs. 4 and 5) together with several other species classified in section *Discolor* (Gerlach and Nirenberg, 1982). The topology inferred from the combined dataset was unchanged when rooted with sequences of *F. oxysporum*, a more distant outgroup (Fig. 5).

Morphological analysis of the Quorn mycoprotein strains revealed that they are all aconidial colonial mutants except for NRRL 26139 (= ATCC 20334 = IMI 145425). This strain produced three cell types diagnostic of *F. venenatum* (Nirenberg, 1995) on SNA agar when grown under continuous black light or in complete darkness at 25°C (Table 2): (1) mostly 5-septate, falcate sporodochial conidia with a pointed apical cell and a pedicellate basal cell (Fig. 6A), (2) conidiophores with primarily doliform monophialides, and (3) chlamydospores typically in recurved terminal chains (Fig. 6B). Conidia produced by strain NRRL 26139 were longer than NRRL 26228 (46.0 vs 44.2 μm, $P < 0.01$) when averaged across growth under black light and complete darkness. Overall, conidia produced under black light were longer than those produced in the dark (46.5 vs 42.3 μm, $P < 0.01$); however, the interaction of strain by light was not significant ($P > 0.10$) and no effects of strain or light source were found for conidial width.

Preliminary runs in SIM revealed that trichothecene mycotoxins were absent in the uninoculated negative control, the three commercial Quorn mycoprotein products (pieces, mince, and quarter pounders; Marlow Foods Limited), and the wild-type Quorn strain NRRL 26139

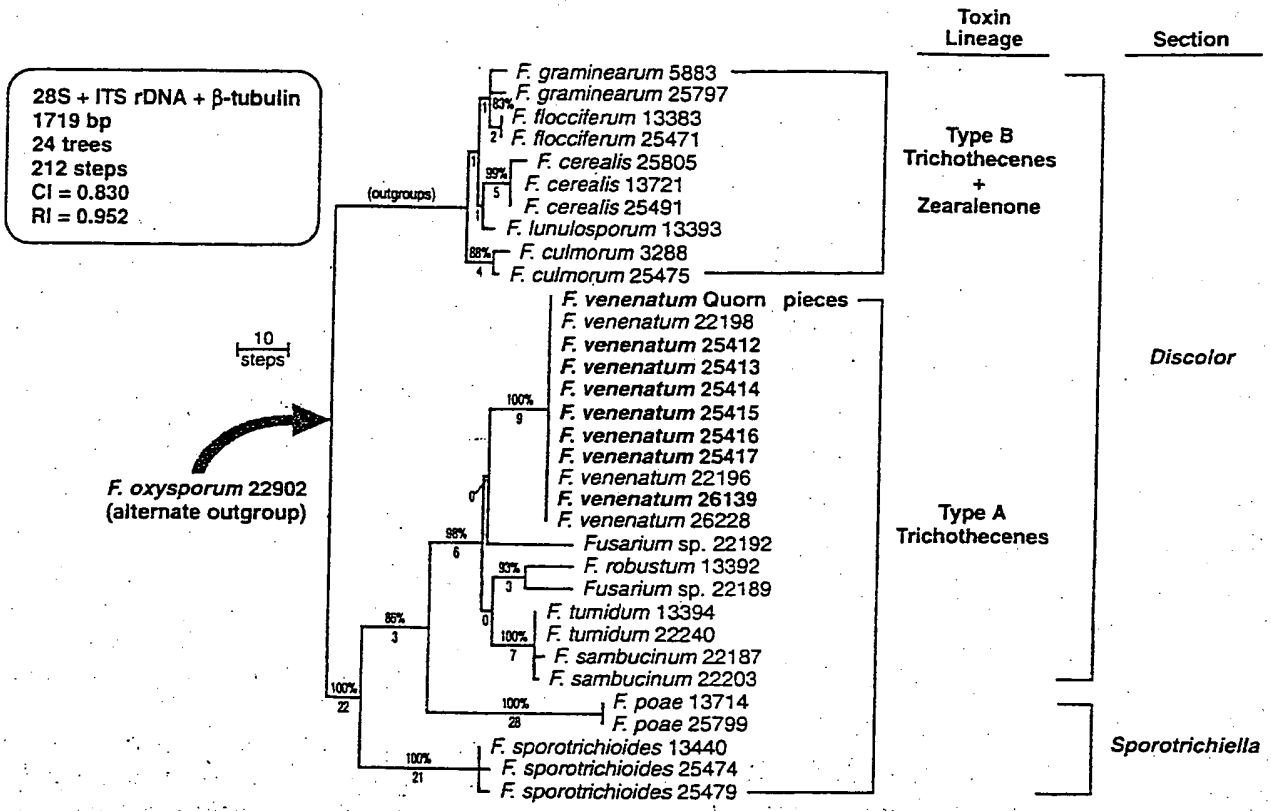


FIG. 5. One of 24 equally most-parsimonious trees based on combined nuclear 28S rDNA, ITS rDNA, and β-tubulin gene sequence data. Bootstrap percentages above 50% (above internodes), edge length, and decay indices (below internodes) are indicated. Note that the traditional, morphology-based sectional classification is nonmonophyletic. The shaded arrow indicates where the root of an alternate, more distant outgroup species, *F. oxysporum*, connects to the tree. That this more distant outgroup does not alter the topology provides strong evidence that the *F. graminearum* clade is an appropriate outgroup to the *F. venenatum* clade. All tree statistics were calculated excluding *F. oxysporum*. Quorn and Quorn strains are in bold type.

(Table 3). However, the other two culture materials contained high levels of several toxins, and a full scan (50–650 *m/z* per second) was used to further identify the mycotoxins and to estimate their concentration. SCIRP, 15AC-SCIRP, DAS, and 4-MAS were identified from rice inoculated with Quorn strain NRRL 25416 (= IMI 154209), but at low levels relative to strain NRRL 22198 *F. venenatum* (Table 3 and see Fig. 7).

DISCUSSION

Data from three sources (molecular phylogenetic, morphological, and mycotoxin analyses) presented in this study independently indicate that the Quorn mycoprotein strains previously identified as *F. graminearum* are *F. venenatum*. All of the Quorn strains formed an exclusive clade with reference strains of *F. venenatum*, including the ex-type strain NRRL 26228, within the ingroup in gene trees inferred from the three regions sequenced and analyzed phylogenetically. Reference strains of *F. graminearum*, in contrast, were nested within the outgroup species in these same gene trees. Topological concordance of gene trees inferred from the three regions sequenced suggests that each reflects the same underlying species phylogeny. Our finding that most of the phylogenetic signal in the combined dataset is contributed by the β-tubulin locus is consistent with previous studies on *Fusarium* (O'Donnell, 1997; O'Donnell and Cigelnik, 1997) which found that the nuclear 28S rDNA and ribosomal ITS region lack sufficient resolution for distinguishing closely related species within this genus. Low measures of clade stability on many nodes and branches, as assessed by bootstrapping and decay analysis, indicate that further testing of the species phylogeny is required via phylogenetic analysis of se-

quences from additional rapidly evolving nuclear genes that possess numerous and/or large introns as in the β-tubulin gene. Results of the current study are concordant with previous reports that infrageneric sections within *Fusarium* are nonmonophyletic (O'Donnell, 1996; O'Donnell and Cigelnik, 1997; Mulé *et al.*, 1997). Data presented here indicate that *F. sporotrichioides* and *F. poae* (section *Sporotrichiella*) form a paraphyletic grade with respect to the remaining ingroup species which are classified in section *Discolor*. That this topology was unaltered even when sequences from a more distant outgroup (*F. oxysporum*) were used to root the tree indicates that the type B trichothecene-producing *F. graminearum* clade is an appropriate outgroup to the type A trichothecene-producing *F. venenatum* clade.

Microscopic examination of Quorn strain NRRL 26139 revealed that it is morphologically similar to the ex-type strain of *F. venenatum* NRRL 26228 (Table 2), a species recently segregated from *F. sambucinum* sensu lato (Nirenberg, 1995). As is characteristic of *F. venenatum*, NRRL 26139 produces sporodochial conidia that are shorter and wider than those produced by strains of *F. graminearum* (Gerlach and Nirenberg, 1982). Further, in contrast to the cylindric to subcylindric monophialides in *F. graminearum*, those of Quorn strain NRRL 26139 are mostly doliform as in *F. venenatum* (Table 2). Last, the recurved terminal chains of chlamydospores produced by NRRL 26139 are typical of *F. venenatum*, while those produced in *F. graminearum* are primarily intercalary in hyphae when present (Gerlach and Nirenberg, 1982).

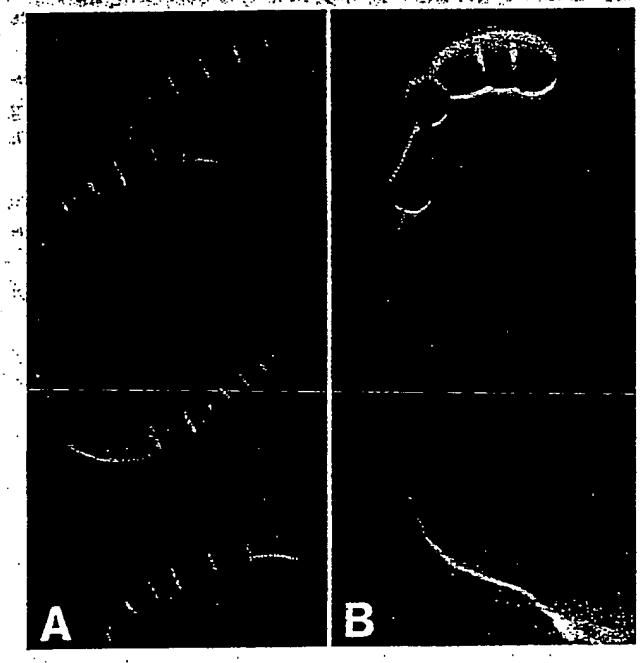
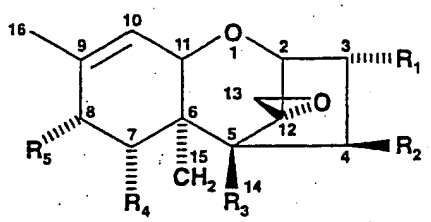


FIG. 6. NRRL 26139 *F. venenatum* viewed and photographed using differential interference optics from cultures grown on SNA agar under continuous black light at 25°C. (A) 5- and 6-septate, falcate sporodochial conidia with a pointed apical cell and a pedicellate. basal cell. (B) Chlamydospores produced in a recurved terminal chain. Scale bar = 20 μm, for A and B.



Trichothecene	R_1	R_2	R_3	R_4	R_5	MW	TMS MW
SCIRP	OH	OH	OH	H	H	282	498
4-MAS	OH	OAC	OH	H	H	324	468
15AC-SCIRP	OH	OH	OAC	H	H	324	468
DAS	OH	OAC	OAC	H	H	366	438

FIG. 7. Structures of the four trichothecene mycotoxins identified (see Table 3). TMS, trimethylsilyl ester derivatives of trichothecenes.

Results of the mycotoxin analysis are concordant with the molecular phylogenetic and morphological analyses which collectively indicate the Quorn mycoprotein fungus is *F. venenatum* rather than *F. graminearum*. *F. venenatum* NRRL 22198 (= BBA 64478), previously shown to produce the type A trichothecene toxin diacetoxyscirpenol (Altomare *et al.*, 1995; Schmidt *et al.*, 1995; Thrane and Hansen, 1995), and Quorn strain NRRL 25416 (= IMI 154209) were able to synthesize the same four type A trichothecene mycotoxins (scirpentriol, 15-acetoxyscirpenol, diacetoxyscirpenol, and 4-monoaceytoxyscirpenol); however, the Quorn strain produced toxins at a much lower level than NRRL 22198. Reference strains of *F. graminearum*, in contrast, produce zearalenone and only type B trichothecenes such as deoxynivalenol (Marasas *et al.*, 1984). Failure to detect mycotoxins in all three commercial Quorn mycoprotein products tested at the 0.5 ppm detection level indicates either that toxins are not induced during culturing or that they are removed during subsequent processing of the mycoprotein. Nevertheless, given the potential of Quorn strain NRRL 25416 *F. venenatum* to elaborate four type A trichothecenes in a rice-inoculated culture, careful monitoring of all commercial mycoprotein should be conducted routinely to ensure that mycotoxin-contaminated Quorn products do not reach the market for human consumption.

Finally, the molecular phylogenetic analysis highlights the importance of investigating the evolution of *Fusarium* toxins within the context of a robust species-level phylogeny. The two toxin lineages resolved in the present study, a deoxynivalenol/zearalenone clade comprising the outgroup species and a DAS/T-2 toxin clade corresponding to the ingroup, have been reported elsewhere (Mulé *et al.*, 1997; O'Donnell, 1997). Although mycotoxin profiles have not been determined for all of the species included in this study, concordance of type A and type B trichothecene production with the ingroup and outgroup species lineages, respectively, provides a testable hypothesis for predicting toxin production and for studying the evolution of mycotoxins within a phylogenetic context. As evidenced by the molecular identification of the Quorn mycoprotein products and morphologically degenerate Quorn strains as *F. venenatum*, phylogenetics can exploit of the wealth of nucleotide data in order to investigate species limits, and these same discrete data can be used to develop molecular diagnostics for the rapid detection and accurate identification of all fusaria.

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